BIOTRANSFORMATION POTENTIAL OF PHYTOSTEROLS IN BIOLOGICAL TREATMENT SYSTEMS UNDER VARIOUS REDOX CONDITIONS

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SUMMARY

Phytosterols are naturally occurring compounds which regulate membrane fluidity and serve as hormone precursors in plants. They also have the potential to cause endocrine disturbances in aquatic animals at concentrations as low as 10 μg/L. Wastewaters from several industries which process plant matter can contain phytosterols at concentrations in excess of the above-stated level. The most common phytosterols are campesterol, stigmasterol and β-sitosterol, with β-sitosterol being the most abundant in most plant species. Despite their endocrine disruption potential, very little is known about phytosterol physical properties and their biotransformation potential in biological treatment systems.

Untreated pulp and paper mill wastewaters have reportedly and typically 0.3 – 3 mg/L total phytosterols. Phytosterols are released from wood during the pulping process and a small fraction enters the wastewater stream during washing of the pulp. Aerated stabilization basins (ASBs) are common biological treatment systems in North American pulp and paper mills. ASBs are large open lagoons which use tapered surface aeration to remove COD and prevent sulfate reduction in the water column. Wastewater solids and biomass settle to the bottom of the lagoons where anaerobic conditions exist. Phytosterols are sparingly soluble and hydrophobic compounds which are typically adsorbed to dissolved and particulate organic matter in aquatic systems. Therefore, phytosterols may be exposed to aerobic or anaerobic environments depending on their solubility and solid-liquid partitioning behavior.

The overall objective of this research was to systematically and quantitatively assess the biotransformation potential of phytosterols in biological treatment systems and to examine conditions leading to reduction of these compounds in wastewater effluent streams. The specific objectives of this research were:

1. Investigate the intrinsic aqueous solubility of phytosterols and the phase distribution of phytosterols in biological treatment systems.
2. Investigate the biotransformation potential of phytosterols in biological systems under various redox conditions (i.e., from fully aerobic to completely anaerobic).
3. Investigate the phase distribution and removal of phytosterols in a continuous-flow, multiple redox zone system.

The aqueous solubility of cholesterol and a phytosterol mixture was measured at 22°C by continuous circulation through a sterol-loaded sand column. The solid-liquid phase partitioning behavior of individual sterols in the presence of pulp mill wastewater solids or aerobic biomass was assessed using batch adsorption and desorption assays. The effect of pH on phase partitioning was also investigated.

The biotransformation potential of phytosterols was investigated using batch assays under aerobic, nitrate-reducing, sulfate-reducing and methanogenic conditions. Cultures used in the batch assays were developed from ASB mixed liquor and sediment and fed untreated pulp mill wastewater. A continuous-flow system was developed to investigate the removal and phase distribution of phytosterols in a simulated ASB.

The results of this research showed that phytosterols are sparingly soluble with aqueous solubility below 1 μg/L when present as a mixture. Phytosterols have a strong affinity to adsorb to solids and dissolved organic matter. The affinity for aerobic biomass

was greater than for wastewater solids. The stigmasterol desorption rate and extent from wastewater solids increased with an increase in pH from 5 or 7 to 9.

Phytosterols were biotransformed under aerobic conditions but not under sulfate-reducing or methanogenic conditions by stock cultures developed in this study. Biotransformation under nitrate-reducing conditions could not be confirmed conclusively. The continuous-flow system was successful in removing 72 to 96% of phytosterols. Biotransformation accounted for 23, 14 and 41 % of campesterol, stigmasterol and β-sitosterol removal, respectively. Phytosterols accumulated in the reactor sediment and accounted for 97 % of the total phytosterols remaining in the system.

Phytosterols can be removed from wastewater streams during biological treatment by a combination of biotransformation and solids partitioning and control of system pH, DO and available carbon and energy sources can increase the degree of phytosterols removal. The results of this research can be used to engineer effective biological treatment systems for the removal of phytosterols from pulp mill wastewaters and other phytosterol-bearing wastewater streams.

CHAPTER 1

INTRODUCTION

Sterols are lipids that serve to regulate membrane fluidity and cross membrane transport in eukaryotic organisms; they also serve as precursors to hormones in plants and animals. Although cholesterol is the predominant sterol found in animals, plants can contain over 200 different sterols, which are collectively known as *phytosterols*. Structures of some common sterols are given in Figure 1.1.

Endocrine system disturbances have been observed in fish downstream of pulp and paper mill wastewater outfalls. Phytosterols are suspected of being a contributor to the endocrine disrupting potential of pulp mill effluents due to their structural similarity to steroidal hormones. Exposure to phytosterols affected steroid production (Gilman et al., 2003; Leusch and Maclatchy, 2003) and induced vitellogenin production in aquatic organisms (Christianson-Heiska et al., 2007; Orrego et al., 2009; Hewitt et al., 2008; Ellis et al., 2003). There is also evidence that some of the observed endocrine disruption is due to microbial transformation products of the phytosterols rather than the phytosterols themselves (Denton et al., 1985). Due to their ubiquity in eukaryotic organisms, phytosterols can be found naturally in both freshwater and marine sediments (Schwendinger and Erdman, 1964). However, industrial activities can elevate aqueous concentrations of phytosterols to unnaturally high levels near plant outfalls, increasing the risk of endocrine disturbances to aquatic organisms.

Wastewater treatment systems have varying levels of effectiveness at removing phytosterols. Activated sludge units at pulp mills have been found to have higher sterol

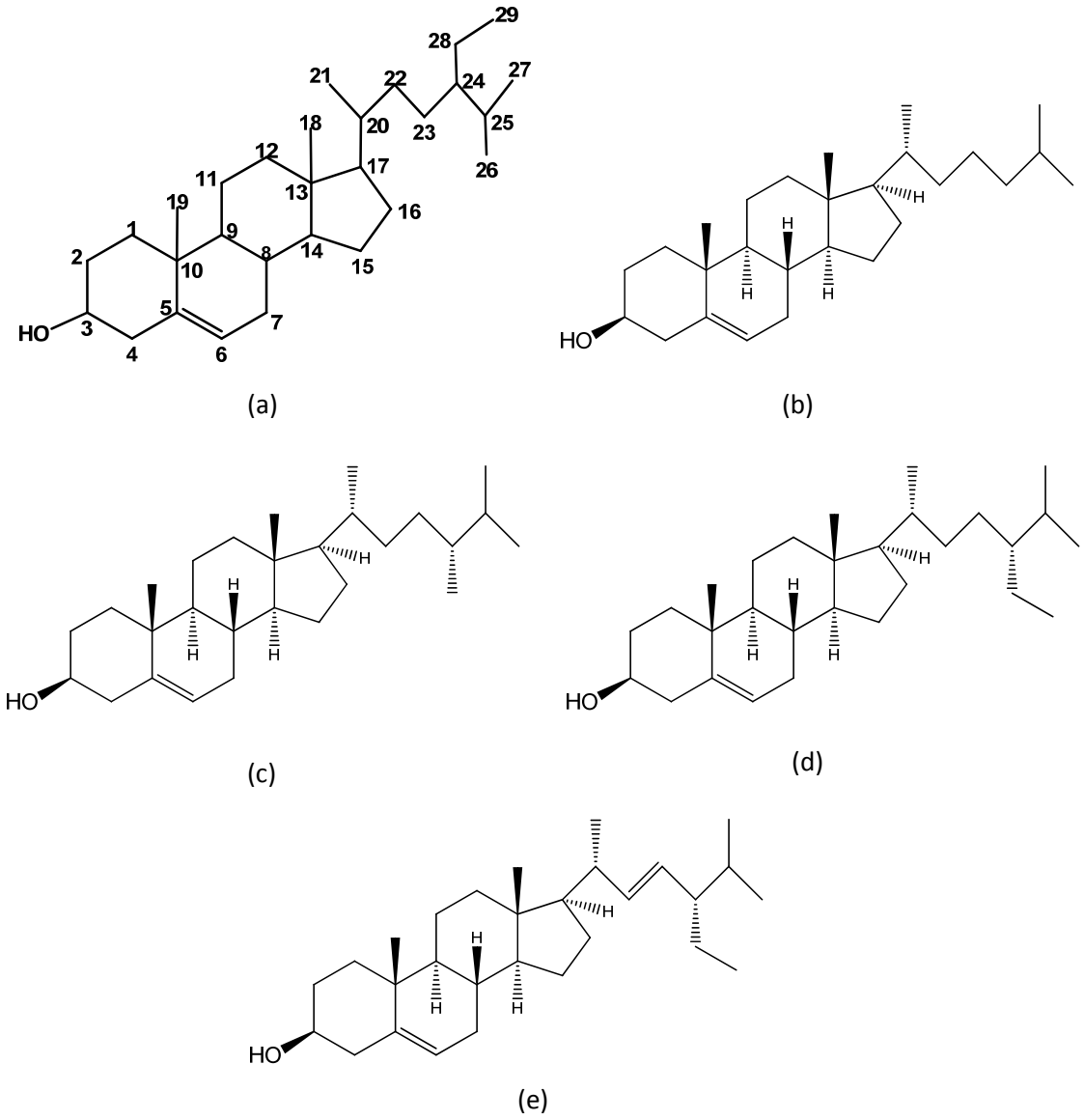

Figure 1.1. Sterol structures: (a) numbered sterol skeleton, (b) cholesterol, (c) β-sitosterol, (d) campesterol, (e) stigmasterol.

removal efficiencies than aerated stabilization basins (ASBs). However, stigmasterol concentrations were found to increase across ASBs by an unidentified mechanism (Cook et al., 1997). The reason for the different treatment efficiencies is not fully understood. ASBs, due to incomplete mixing, contain several redox zones ranging from aerobic in the upper water column to sulfate-reducing and methanogenic in the sediment. Phytosterols have been found to be readily degradable aerobically, but their biodegradation potential under anoxic and anaerobic conditions is poorly understood.

The biodegradation potential of phytosterols depends on their bioavailability. If sorbed to particulate materials or otherwise sequestered, their bioavailability can be greatly reduced. Phase partition constants, such as the octanol-water and liquid-solid partition coefficients, have not been experimentally determined for phytosterols.

The overall objective of this research was to systematically and quantitatively assess the biotransformation potential of phytosterols in biological treatment systems and to examine conditions leading to reduction of these compounds in wastewater effluent streams. The specific objectives of this research were:

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2. Investigate the biotransformation potential of phytosterols in biological systems under various redox conditions (i.e., from fully aerobic to completely anaerobic).
3. Investigate the phase distribution and removal of phytosterols in a continuous-flow, multiple redox zone system.

CHAPTER 2

BACKGROUND

2.1. Natural Occurrence and Anthropogenic Sources

Sterols are naturally occurring lipids that regulate fluidity of cell membranes in eukaryotes and also participate in membrane transport. In both plants and animals, sterols are precursors for the synthesis of sex and other hormones. Animal sterols consist almost exclusively of cholesterol with a small amount of lanosterol present in some tissues. Plants, on the other hand, contain over 200 different sterols which are collectively known as *phytosterols* (Moreau et al., 2002). The most prevalent phytosterols are β-sitosterol, stigmasterol, and campesterol with β-sitosterol being the most common in pulp mill wastewater (Fernandes et al., 2003). Cholesterol constitutes 1-2% of total sterols in most plants but some species can contain 5% or more (Moreau et al., 2002). Although cholesterol is not considered a phytosterol, it is being included as a model compound in this research due to the published information related to cholesterol degradation and the commercial availability of cholesterol degradation products. Relatively little research has been conducted into phytosterol degradation, and phytosterol degradation products are not commercially available.

In vivo, phytosterols can exist in both free and conjugated forms. Conjugated phytosterols do not perform the same membrane regulatory functions as free sterols, but instead aid in sterol transport and are thought to function as a reserve pool of phytosterols. The four main classes of phytosterol conjugates are fatty acyl sterol esters, steryl glucosides, acylated steryl glucosides and hydroxycinnamate steryl esters.

Conjugated sterols are more hydrophobic than free sterols and tend to accumulate on hydrophobic surfaces when present in aquatic environments. Sterol conjugates are not expected to be present in biological wastewater treatment systems at environmentally significant aqueous concentrations and thus are beyond the scope of this research.

Vegetable oils generally contain 1-5 mg/g total phytosterols; however, some can contain much higher concentrations, e.g. corn oil can contain 8 to >15 mg/g total phytosterols (Piironen et al., 2000). Soybean oil deodorizer distillate, a byproduct of soybean oil refining, is a major source of phytosterols for the pharmaceutical industry, where they are used as starting material for the synthesis of steroid hormones. The total phytosterol content of soybeans is 0.3 – 0.6 mg/g (Mohamed et al., 2001). Each vegetable oil also has a characteristic profile of phytosterol concentrations. Because free phytosterols are not degraded during biodiesel production, sterol profiles have been proposed as a means of tracking the source of biodiesel spills (Spikmans et al., 2011). Biodiesel derived from canola and soybean oil contains 5.92 and 1.49 mg total sterols/g (Spikmans et al., 2011). Other example phytosterol concentrations are 0.63 mg free sterols/g for wheat straw, a paper-making feedstock (Qin et al., 2009) and 0.077 μg cholesterol/L and 86 μg stigmasterol/L for milk processing wastewater (Verheyen et al., 2011).

All sterols are susceptible to enzymatic and auto-oxidation. Enzymatic oxidation tends to affect the side-chain whereas non-enzymatic oxidation affects the ring system (Smith, 1996). Auto-oxidation is initiated by reactive oxygen or nitrogen species alone or in response to UV irradiation or heat. A hydrogen atom is abstracted at C7 by the reactive species and molecular oxygen is incorporated resulting in the formation of a

hydroperoxide which can then decompose into a 7-hydroxysterol or 7-ketosterol (Ryan et al., 2009). Tertiary carbons in the side chain are also vulnerable to attack by reactive oxygen species resulting in the formation of C20 or C25 hydroperoxides. Reduced phytosterols, analogous to cholestanol, have been found to be less susceptible to oxidation than free phytosterols (Soupas et al., 2007). Cholesterol oxidation products (COPs) in vivo are associated with atherosclerosis, Alzheimer's disease and some types of cancer (Jusakul et al., 2011). COP:cholesterol ratios in healthy adults are in the range of 0.02 – 1.0% (Linseisen and Wolfram, 1999). The oxidation of phytosterols in stored foods and heated vegetable oils, as well as the generation of phytosterol oxidation products (POPs) in vivo, has been the subject of several studies (Otaegui-Arrazola et al., 2010; Garcia et al., 2008; Nielsen et al., 1996; Soupas et al., 2007).

2.2. Chemical and Physical Properties

Phytosterols are built around the four ring structure of cyclopentanoperhydro-phenanthrene, common to all steroids (Figure 1.1). Phytosterols can be classified based on the number of methyl groups on C4. The most common phytosterols, as well as cholesterol, are desmethyl (no methyl) sterols and contain a double bond between C5 and C6. They have a hydroxyl group on C3 and a hydrocarbon chain at C17 which terminates in an isopropyl group. β-sitosterol, campesterol and stigmasterol are branched at C24, but cholesterol is not. It is the branching pattern and saturation of the side aliphatic chain that differentiates between the common sterols. Cholesterol has the simplest side chain structure and stigmasterol the most complex (Figure 1.1.).

Experimental aqueous solubilities for phytosterols are unavailable, but they are reported to be sparingly soluble in water at near neutral pH with expected aqueous

solubility of below 10 µg/L. The aqueous solubility of cholesterol was experimentally determined to be 95 µg/L at 30°C (VCCLAB). The estimated pK_a of the phytosterol hydroxyl group is above 15 (Table 2.1); thus, phytosterols exist almost exclusively in their neutral form under environmentally relevant conditions and their intrinsic sorption behavior and bioavailability are not expected to be affected by pH. Estimated log K_{ow} values are above 8 and vapor pressures are estimated to be below 1.5 x 10^{-11} Torr (Estimations obtained from SciFinder which cites ACD/Labs software as their source).

Table 2.1. Selected properties of target sterols.

Sterol	**MW**	**Aq. Solubility @ 25°C pH=7 (µg/L)**	**Vapor P @ 25°C[a] (Torr)**	**Log K_{ow}[c]**	**pK_a[a]**
β-sitosterol	414.71	0.9[a], 0.5[b]	3.53E-12	9.65	15.03±0.7
Stigmasterol	412.69	1.6, 1.2	3.84E-12	9.43	15.03±0.7
Campesterol	400.68	2.0, 1.8	1.23E-11	9.16	15.14±0.7
Cholesterol	386.65	3.3, 3.6	2.95E-11	8.74	15.03±0.7

[a] SciFinder (ACD Labs software estimation); [b] EPI Suite WATERNT, [c]EPI Suite KOWWIN

2.3. Biotransformation

2.3.1. Observations in Biological Systems

The removal of phytosterols from wastewaters by full-scale pulp mill treatment systems has been examined by several studies (Mahmood-Khan and Hall, 2003; Cook et al., 1997). Activated sludge units are able to remove > 90% of all phytosterols. ASBs are also able to remove a high fraction of most phytosterols. Stigmasterol, however, was found by one study to increase across all five ASBs sampled, and in some cases the concentration of stigmasterol was > 200% greater in the ASB effluent than in the influent (Cook et al., 1997). Removal in these systems may be due to biotic and abiotic transformation of the phytosterols or to adsorption and sequestration to sludge and sediment. Phytosterols have been found to accumulate in sludge and sediment. Pulp mill activated sludge contained 10-32 mg/L total sterols (Mahmood-Kahn and Hall, 2003). Biosolids from a Japanese domestic wastewater treatment plant contained 19.1-157 mg/kg cholesterol, 50.8-200 mg/kg β-sitosterol and 2.76-17.4 mg/kg stigmastanol per unit mass of organic carbon (Citulski and Farahbakhsh, 2010). Sterols were found in aquatic sediments, both freshwater and marine, at several hundred parts per million of organic carbon (Scwendinger and Erdman, 1964).

A few laboratory studies have also been performed to assess sterol removal by simulated wastewater treatment systems. Stigmasterol increased across a simulated aerobic lagoon when the sterol loading rate was 0.2 mg/L·d, but removal was >90% when the loading rate was 0.6 mg/L·d (Chamorro et al., 2009). A continuous flow anaerobic filter removed 77-100% of β-sitosterol and 87-95% of stigmasterol when they were added in a 1:1 ratio at combined loading rates of 1.6 to 2.7 mg/L·d (Vidal et al., 2007).

2.3.2. Pathways/Mechanisms

The initial reactions in the degradation of phytosterols and cholesterol are thought to be catalyzed by the same enzymes due to their structural similarity, but the degradation of cholesterol has been studied in greater detail than phytosterols. The complete mineralization of cholesterol has been observed under both aerobic and denitrifying conditions (Harder and Probian, 1997). The first step in the cholesterol degradation pathway is oxidation to cholest-5-en-3-one followed by isomerization to cholest-4-en-3-one (Figure 2.1). These reactions are catalyzed by either of two classes of dual-function enzymes in microorganisms: cholesterol oxidases, which require molecular oxygen as electron acceptor, or cholesterol dehydrogenases/isomerases, which use NAD^+ or $NADP^+$ as co-factors (Uhia et al., 2011). Side chain degradation is then initiated by the hydroxylation of C27 or C25 followed by ring cleavage (Malaviya and Gomes, 2008; Chiang et al., 2008). *Sterolibacterium denitrificans* is able to completely degrade cholesterol under both aerobic and denitrifying conditions using water as an oxygen source (Chiang et al., 2008) and is one of only two identified strains capable of mineralizing cholesterol under denitrifying conditions, the other one being strain 72Chol.

Cholesterol metabolism is believed to be a key factor in the virulence of *Mycobacterium tuberculosis*. To try and elucidate the specific pathways used by *M. tuberculosis*, the genes involved in cholesterol degradation by other actinomycetes, notably *Rhodococcus jostii* RHA1, have been studied in detail (Van der Geize et al., 2007). The anaerobic degradation of cholesterol, however, has received less attention. The hydrogenation of cholesterol to cholestanol is known to occur in the mammalian gut. In experiments with sewage sludge and lake sediment, the only observed transformation

of cholesterol was a hydrogenation of 0.5% of the original cholesterol mass to cholestanol over three months of incubation (Gaskell et al., 1975). An early experiment resulted in no transformation of cholesterol under sulfate-reducing conditions (Taylor et al., 1981).

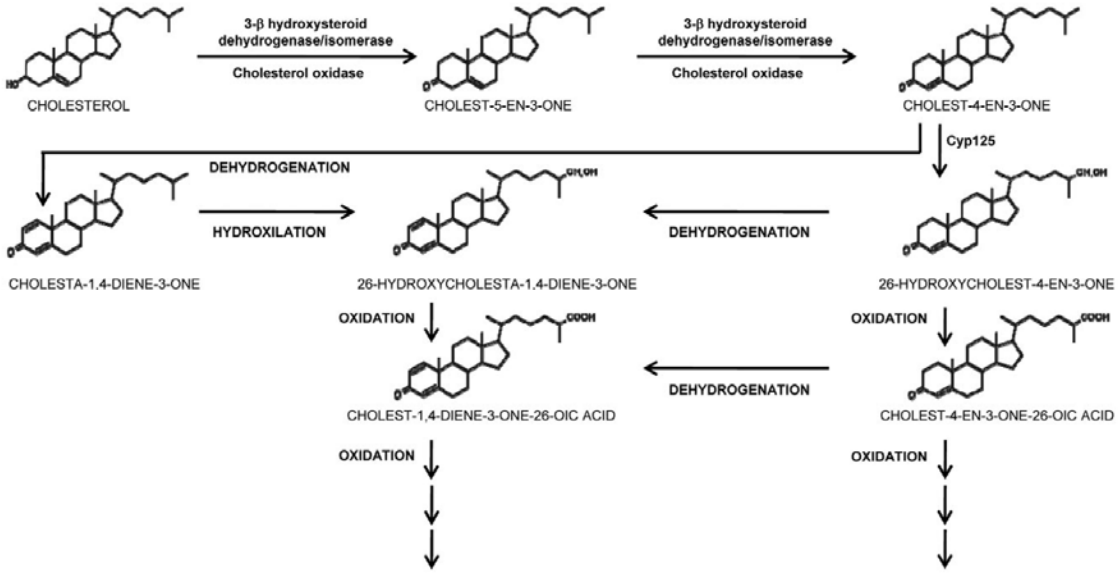


Figure 2.1. Proposed initial steps in cholesterol degradation by *Mycobacterium smegmatis* (Uhia et al., 2011).

The biodegradation of phytosterols is less well understood. Many species are capable of transforming phytosterols aerobically (Mahato and Garai, 1997) and sterols are widely used in the pharmaceutical industry as starting materials for the synthesis of steroids (Figure 2.2). These industrial processes are dependent on microorganisms that selectively degrade the sterol side chain but leave the ring structure intact. Frequently, the species used are mutants but many wild type species are also used. The degradation pathways for the transformation of phytosterols have received less attention than those for cholesterol but are believed to follow much the same path. However, some discrepancies have been found in the genes involved. For instance, in *Rhodococcus rhodocrous* 43269, the gene *fad*19 is necessary for side chain degradation of C24 branched sterols but not of cholesterol (Wilbrink et al., 2011).

The degradation of phytosterol oxidation products has received little attention and thus there are few available references. Degradation of 7-ketocholesterol by *Rhodococcus jostii* RHA1 involved 363 genes which were differentially expressed compared to during degradation of cholesterol. However, several key genes involved in cholesterol degradation were upregulated during degradation of 7-ketocholesterol suggesting a shared pathway (Mathieu et al., 2010).

2.4. Endocrine Disruption

Endocrine disruption includes interference with any system that is regulated by hormones. Phytosterols interfere with sex hormone regulated systems in fish and cause both estrogenic and androgenic effects. Sex steroid synthesis takes place inside the mitochondria by the conversion of cholesterol. Phytosterols are believed to decrease sex steroid levels in fish by reducing cholesterol transport across the mitochondrial

membrane (Leusch and MacLatchy, 2003) as well as weakly binding to estrogen receptors (Tremblay and Van der Kraak, 1998). Brook trout implanted with a 100 µg/g dose of a phytosterol mixture showed decreased intra-mitochondrial cholesterol and plasma testosterone levels after 20 days (Gilman et al., 2003). A similar experiment in goldfish also resulted in decreased reactive cholesterol levels (Leusch and MacLatchy, 2003). Exposure to β-sitosterol has been shown to induce vitellogenin production in

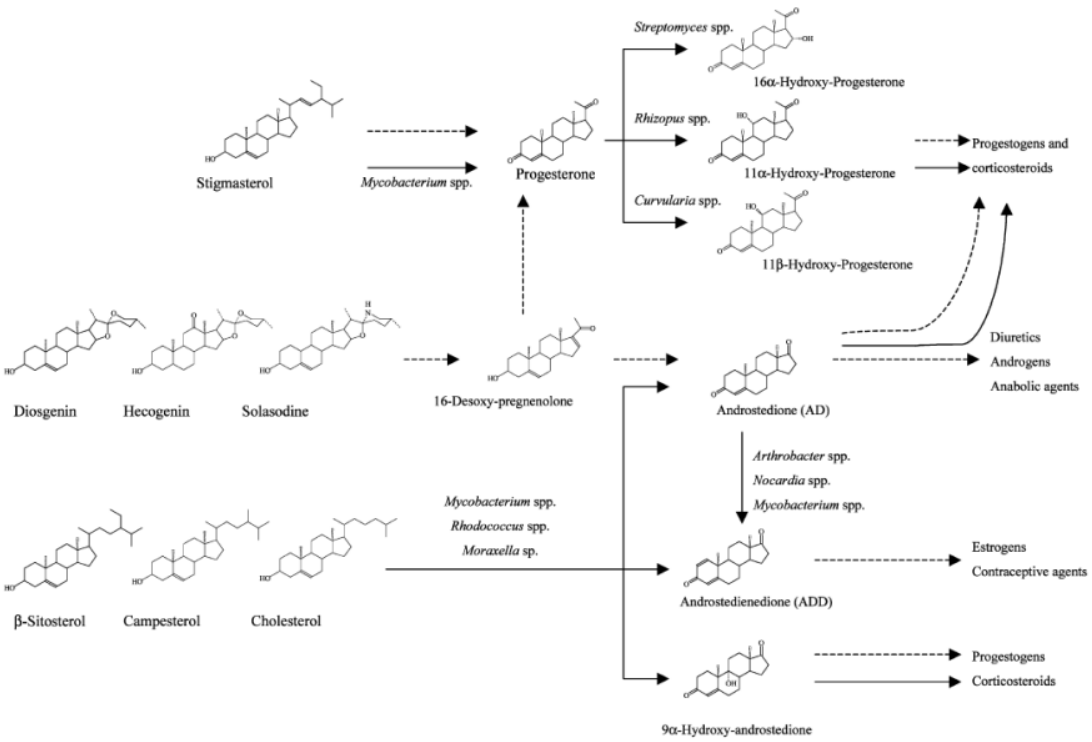


Figure 2.2. Example microbial transformations of sterols used by the pharmaceutical industry (Fernandes et al., 2003). Solid arrows represent biological transformations and dotted lines represent chemical transformations.

juvenile rainbow trout liver cells in vitro (Tremblay and Van der Kraak, 1998). Vitellogenin is a protein found in oocytes that is not normally produced by male or juvenile fish. Male zebrafish exposed to an aqueous concentration of 10 µg phytosterols/L were induced to produce vitellogenin (Christianson-Heiska et al., 2007). In the same study, male zebrafish in vivo and female zebrafish ovarian follicles in vitro were exposed to an aqueous concentration of 100 µg/L of an oxidized phytosterol mixture. Both exposures resulted in increased testosterone production after exposure to the oxidized phytosterols. In a reproductive study on brook trout, fish of both sexes were exposed to 10 and 20 µg/L of a phytosterol mixture for 4.5 months before spawning. Control eggs were fertilized and incubated in clean water. Exposed fish had dose-dependent increased egg mortality and incidence of deformed or diseased larvae (Lehtinen et al., 1999). There is also evidence that phytosterol transformation products induce androgenic changes in mosquitofish (Denton et al., 1985). As mentioned above, several microbial species are capable of transforming cholesterol and phytosterols into steroid hormones. *Lactobacillus bulgaricus* is known to convert cholesterol to testosterone in the presence of glucose as cosubstrate (Kumar et al., 2001).

2.5. Pulp Mill Treatment Systems

2.5.1. Overview

Phytosterols are liberated from wood as a result of the pulp and paper making process along with other lipophilic extractives. Extractives are released during alkaline pulping when lignin is broken down and solubilized to leave the cellulose fibers. Phytosterols and other extractives, which include terpenes, phenolics, fats, fatty acids and resin acids, become concentrated in the pulping solution which is known as black liquor.

Black liquor is drained from the pulping chamber after pulping and burned for energy recovery. A small amount of the liquor remains in the pulp and is gradually washed out of the pulp during further processing. Washing occurs after several stages of the pulp making process including alkaline pulping, oxidation of the pulp, alkaline extraction and pulp bleaching. Phytosterols and other extractives are transported with the wash water into the wastewater sewers.

The concentration of phytosterols in pulp mill wastewaters can vary depending on the pulping method used, the species of wood being pulped, and the amount of water being used by the mill. Hardwood species such as Aspen and Birch contain higher steryl ester levels than softwood species (e.g., pine). Spruce wood chips contain 0.16 mg free phytosterols/g dry weight and 0.23 mg steryl esters/g dry weight (van Beek et al., 2007). Eucalyptus that has been aged for 20 days and stripped free of bark contains 4.2 mg total free sterols/g dry wood (Silverio et al., 2008). Other nonwoody lignocellulosic crops also contain significant quantities of phytosterols and phytosterol conjugates. Flax, hemp, sisal, and abaca each were found to contain 92, 36, 20 and 25 mg free sterols/100 g dry weight (Marques et al., 2010). Typical total phytosterol concentration in untreated pulp mill wastewater varies from 0.3 to 3.4 mg/L (Xavier et al., 2009).

As a general rule, softwood species contain more lipophilic extractives than hardwood species and only softwood species contain resin acids. The total extractives content is approximately 2.1% of dry weight for softwoods and 0.8% of dry weight for softwoods. Resin acids are acutely toxic to rainbow trout in the range of 0.4 - 1.1 mg resin acids/L (Leach and Thakore, 1976), have high antiestrogenic potential (Terasaki et al., 2009) and inhibit methanogenesis (McCarthy et al., 1990). Several phenolic

compounds have significant estrogenic activity (Terasaki et al., 2005). Because of the presence of multiple classes of compounds with potential endocrine disrupting capability, it is difficult to assign the overall endocrine disrupting potential of pulp mill wastewater to only one class of compounds.

2.5.2. Aerated Stabilization Basins (ASBs)

Secondary treatment of pulp mill wastewater is required because of high chemical oxygen demand (COD) load and toxicity. Aerated stabilization basins (ASBs) are commonly used by pulp mills, especially in older North American mills where space has not been an issue. These basins use tapered aeration provided by surface aerators to supply oxygen for COD removal and the prevention of sulfate reduction, which can lead to odor issues. The aerators also provide mixing of ASBs at a flow regime that is between completely mixed and plug-flow. The uneven flow as well as the buildup of sulfate-rich sediment leads to the presence of multiple redox environments within the same ASB, ranging from fully aerobic on the top of the water column to completely anaerobic conditions in the sediment. Periods of disturbance (e.g., storms, sediment dredging) can lead to cycling between the sediment and the water column resulting in redistribution of organic matter and adsorbed contaminants within the ASB, possibly changing the bioavailability of phytosterols. ASBs at pulp mills typically have to be dredged every two years (Mahmood, 2008).

CHAPTER 3

MATERIALS AND ANALYTICAL METHODS

3.1. General Analytical Methods

3.1.1. pH

All pH measurements were performed using the potentiometric method with a ATI Orion Model 370 digital pH meter (Orion Research Inc., Boston, MA) and a gel-filled combination pH electrode (VWR International, West Chester, PA). The meter was calibrated weekly with pH 4.0, 7.0, and 10.0 standard buffer solutions (Fisher Scientific, Pittsburg, PA).

3.1.2. Dissolved Oxygen (DO)

Dissolved oxygen in this study was measured using the luminescent dissolved oxygen method with a Hach HQ40d digital multimeter in conjunction with a LDO101 oxygen probe (Hach Company, Loveland, CO). The instrument was calibrated to water-saturated air at 22°C before each use.

3.1.3. Chemical Oxygen Demand (COD)

COD was measured using the closed reflux, colorimetric method as described in *Standard Methods* (Eaton et al., 2005). An aliquot of 3 mL digestion solution composed of 4.9 g $K_2Cr_2O_7$, 6 g $HgSO_4$, 6 g Ag_2SO_4 and 500 mL H_2SO_4 was transferred to HACH COD digestion vials (HACH Company, Loveland, CO) and then 2 mL of sample was added to the vial. After tumbling the vial for 4-8 times, the content in the vials was

digested at 150°C for 2 hours and then cooled down to room temperature. The absorbance was measured at 620 nm with a Hewlett-Packard Model 8453 UV/Visible spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) equipped with a diode array detector, deuterium and tungsten lamps and a 1 cm path length. Samples were centrifuged and filtered through a 0.45 μm polypropylene membrane filter if the soluble COD was measured, otherwise well-mixed samples were used after appropriate dilution for total COD measurements. All samples were prepared in triplicates and a calibration curve was prepared using 1 g/L standard solution of potassium hydrogen phthalate (KHP).

3.1.4. Ammonia

Ammonia concentration was measured using the distillation method described in *Standard Methods* (Eaton et al., 2005). The samples were centrifuged at 12,000 rpm for 15 minutes and filtered through a 0.2 μm nitrocellulose membrane filter (Fisher Scientific, Pittsburgh, PA). The ammonia distillation was performed using a Labconco distillation apparatus (Labconco Corp., Kansas City, MO) and the distillate was received with a mixed indicator solution of 20g H_3BO_3/L, methyl red 13.3 mg/L and methylene blue 6.67 mg/L. The distillate then was titrated with 0.2 N H_2SO_4 and the ammonia was quantified.

3.1.5. Ethanol

Ethanol was measured with a HP 1100 Series HPLC (Hewlett Packard, Palo Alto, CA) unit equipped with an Aminex HPX-87H ion exclusion column (300 × 7.8 mm)(Bio-Rad, Richmond, CA) refractive index detector (Agilent Technologies, New Castle, DE). A 0.01 N H_2SO_4 solution was used as the mobile phase with a flow rate of 0.6 mL/min and the column was maintained at 65ºC. The samples were centrifuged and the supernatant

was acidified with 0.2 N H_2SO_4 in a 1:1 ratio, and filtered through 0.2 μm membrane filters before the analyses.

3.1.6. Anions

Chloride (Cl^-), nitrite (NO_2^-), bromide (Br^-), nitrate (NO_3^-), phosphate (PO_4^{3-}), and sulfate (SO_4^{2-}) anion concentrations were determined using a Dionex DX-100 ion chromatography unit (Dionex Corporation, Sunnyvale, CA) equipped with a suppressed conductivity detector, a Dionex IonPac AG14A (4x50mm) precolumn, and a Dionex IonPac AS14A (4x250 mm) analytical column. The unit was operated in autosupression mode with 1 mM $NaHCO_3$/8 mM Na_2CO_3 eluent and a flow rate of 1 mL/min. All samples were filtered through 0.2 μm membrane filters prior to injection. The minimum detection limit for each anion listed above was 0.03, 0.02, 0.03, 0.04, 0.02 and 0.05 mM, respectively.

3.1.7. Total and Volatile Solids (TS and VS)

Total solids content of samples were determined according to procedures outlined in *Standard Methods* (Eaton et al., 2005). Samples were weighed in pre-ignited (550^{o}C) and cooled ceramic crucibles using an Ohaus AP250D Analytical Balance (precise to ±0.02 mg up to 52 g, and to ±0.1 mg between 52 and 210 g). The samples were then dried at 105^{o}C for 24 hours in a Fisher Isotemp Model 750G oven. After drying, the crucibles were transferred to a desiccator until cooled, and then the dry weight was measured. If VS were to be determined, the crucibles were transferred to a Fisher Isotemp Model 550-126 muffle furnace and ignited at 550^{o}C for 20 minutes. After ignition, the samples were cooled in a desiccator and the remaining solids weight was measured. TS and VS were then calculated. Total solids were calculated as the difference between the

weight of the crucible after the sample was dried at 105°C and the tare weight of the crucible divided by the sample volume. Volatile solids were calculated as the difference between the weight of the crucible after the sample was dried at 105°C and the weight of the crucible after the sample was burned at 550°C divided by the sample volume.

3.1.8. Total and Volatile Suspended Solids (TSS and VSS)

TSS and VSS were determined according to procedures described in *Standard Methods* (Eaton et al., 2005). Whatman GF/C glass fiber filters (47 mm diameter and 1.2 μm nominal pore size; Whatman, Florham Park, NJ) were washed with deionized (DI) water and ignited at 550^{o}C for 20 minutes in a Fisher Isotemp Model 550-126 muffle furnace before use. The filters were than cooled in a desiccator and weighed. Samples of known volume were filtered through the glass fiber filters. The filters were then rinsed with 10 mL DI water to remove dissolved organics and inorganic salts. The filters containing the samples were dried at 105^{o}C for 90 minutes. After cooling in a desiccator, the dry weight was recorded and the filters containing the dry samples were ignited at 550^{o}C for 20 minutes. After ignition, the samples were cooled down in a desiccator and the weight was measured. TSS and VSS concentrations were then calculated. Total suspended solids were calculated as the difference between the weight of the filter after the sample was dried at 105°C and the tare weight of the filter divided by the sample volume. Volatile suspended solids were calculated as the difference between the weight of the filter after the sample was dried at 105°C and the weight of the filter after the sample was burned at 550°C divided by the sample volume.

3.1.9. Total Gas Production

Total gas production in closed assay bottles and large volume reactors was measured by either the gas-water displacement method or with a VWR Pressure/Vacuum transducer (resolution –1 atm to 1.974 atm with an accuracy of 0.002 atm).

3.1.10. Gas Composition

The gas composition was determined by a gas chromatography (GC) unit (Agilent Technologies, Model 6890N; Agilent Technologies, Inc., Palo Alto, CA) equipped with two columns and two thermal conductivity detectors. Methane (CH_4) and dinitrogen (N_2) were separated with a 15 m HP-Molesieve fused silica, 0.53 mm i.d. column (Agilent Technologies, Inc.). Carbon dioxide (CO_2), hydrogen sulfide (H_2S), nitric oxide (NO) and nitrous oxide (N_2O) were separated with a 25 m Chrompac PoraPLOT Q fused silica, 0.53 mm i.d. column (Varian, Inc., Palo Alto, CA). Helium was used as the carrier gas at a constant flow rate of 6 mL/min. The 10:1 split injector was maintained at 150^{o}C, the oven was set at 40^{o}C and the detector temperature was set at 150^{o}C. All gas analyses were performed by injecting a 100 µL gas sample. The minimum detection limits for CH_4, CO_2, H_2S, NO, N_2O and N_2 was 500, 800, 100, 500, 7 and 50 ppmv, respectively.

3.1.11. Volatile Fatty Acids

VFAs (C_2 to C_7; i.e., acetic, propionic, iso-butyric, n-butyric, iso-valeric, n-valeric, iso-caproic, n-caproic and heptanoic acids) were measured after acidification of filtered samples with a 2.5% H_3PO_4 solution containing 1.5 g/L acetoin as the internal standard (sample:acid, 2:1 volume ratio) using an Agilent 6890 Series GC unit equipped with a flame ionization detector and a 35-m Stabilwax-DA, 0.53-mm I.D. column (Restek, Bellefonte, PA). Samples used for the measurement of VFAs were prepared by

centrifugation at 10,000 rpm for 30 minutes and filtration through 0.22-µm PVDF membrane filters before acidification. The minimum detection limit for each acid mentioned above was 0.25, 0.10, 0.03, 0.02, 0.10, 0.08, 0.02, 0.02, 0.05 mM, respectively.

3.1.12. Ionic Strength

Ionic strength in this study was measured with a Hach HQ40d digital multimeter in conjunction with a CDC401 conductivity probe (Hach Company, Loveland, CO). The instrument was calibrated to a 180 µS/cm standard solution of sodium chloride (85.47 mg/L) before each use.

3.1.13. Total and Soluble Carbohydrates

Total and soluble carbohydrates were measured using the anthrone method (Morris, 1948). The anthrone solution was prepared by dissolving 1 g of anthrone (Sigma Aldrich) in 500 mL 98% H2SO4. Samples for soluble carbohydrates analysis were centrifuged and filtered through a 0.45µm polypropylene membrane filter; otherwise, well-mixed samples were used after appropriate dilution for total carbohydrates analysis. Each sample was digested in glass digestion vials with the 0.2% anthrone solution for 15 minutes in a boiling water bath. Absorbance was measured at 620 nm with a Hewlett-Packard Model 8453 UV/Visible spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) equipped with a diode array detector, deuterium and tungsten lamps and a 1 cm path length. Well-mixed samples were used after appropriate dilution for total carbohydrates measurements. All samples were prepared in triplicates and a calibration curve was prepared using 1 g/L standard solution of glucose.

3.1.14. Total Carbon (TC)

TC measurements were performed using a Shimadzu TOC-5050A Total Organic Carbon Analyzer equipped with SSM-5000A Solid Sample Module (Shimadzu Scientific Instruments Inc., Columbia, MD) equipped with a non-dispersive infrared detector. Samples of known volume were added to ceramic crucibles and dried for 4 hours in an oven set at 105ºC to remove moisture. The ceramic crucibles were then combusted at 900ºC inside the SSM unit using ultra high purity oxygen. The evolved CO_2 was quantified using the response of the NDIR detector. Triplicate measurements were performed for each sample. A calibration curve was prepared using KHP.

3.1.15. Dissolved Organic Carbon (DOC)

DOC measurements were performed using a Shimadzu TOC-5050A Total Organic Carbon Analyzer (Shimadzu Scientific Instruments Inc., Columbia, MD) equipped with a non-dispersive infrared detector for the analysis of total, organic and inorganic carbon of liquid samples. Liquid samples were filtered through 0.2 µm PVDF filters, acidified below pH 2.0 using a 0.2 N HCl solution and purged with CO_2-free air for 2 minutes. Triplicate measurements were performed for each sample using a 25 µL injection volume. Carbon analysis was based on catalytic combustion of the sample at 680^{o}C. A calibration curve was prepared using 1g C/L standard solution of KHP.

3.1.16. Total and Soluble Phenolics

Total and soluble phenolics were measured using the Folin-Ciocalteau method (Waterhouse, 2002). Folin-Ciocalteau reagent (2 N) was purchased from Sigma Aldrich (St. Louis, MO). Samples for soluble phenolics analysis were centrifuged and filtered through a 0.45µm polypropylene membrane filter; otherwise, well-mixed samples were

used after appropriate dilution for total phenolics analysis. Samples were mixed with Folin-Ciocalteau reagent and a 200 g/L sodium carbonate solution then extracted into chloroform using a 100 mg/L methyl-tri-tertbutyl ammonium solution. Absorbance was measured in the chloroform extract at 765 nm with a Hewlett-Packard Model 8453 UV/Visible spectrophotometer (Hewlett-Packard Co., Palo Alto, CA) equipped with a diode array detector, deuterium and tungsten lamps and a 1 cm path length. All samples were prepared in triplicates and a calibration curve was prepared using 1 g/L standard solution of gallic acid (Sigma Aldrich, St. Louis, MO).

3.2. Analysis of Phytosterols and Cholesterol

3.2.1 Chemicals

Cholesterol (≥ 99%) and stigmasterol (~ 95%) were obtained from Sigma Aldrich (St. Louis, MO). A phytosterol mixture isolated from soybeans and assayed as 25.2% campesterol, 25.4% stigmasterol and 41.3% β-sitosterol was purchased from Purebulk.com (Roseburg, OR). Iso-octane (> 99.5%) was purchased from Sigma-Aldrich. Stock solutions were prepared in ethanol (> 99%) at 8, 4 and 4.57 g/L for cholesterol, stigmasterol and the phytosterol mixture, respectively.

3.2.2. General Extraction and Sample Preparation

Routine preparation and extraction of samples with expected individual concentrations of phytosterols and cholesterol above the method detection limit (50 µg/L), was conducted using liquid-liquid extraction with iso-octane. Extraction tubes, pipettes and syringes were washed with iso-octane prior to use. The samples were collected by either decanting the sample into the extraction tube, transferring the sample

with a glass graduated pipette or transferring the sample with a glass syringe and stainless steel needle. Culture tubes (25 mL), sealed with Teflon-coated rubber stoppers and sealed with aluminum crimps were used for extraction. A 2:1 sample:iso-octane volume ratio was used, and extraction was performed by tumbling for ≥ 12 h at 22°C. After extraction, 1 mL of iso-octane from the extraction tube was transferred to a 2 mL autosampler vial using a glass Pasteur pipette and sealed with a PTFE-coated septum and aluminum crimp. Extraction efficiencies from the aerobic and sulfate-reducing stock cultures are given in Table 3.1.

Table 3.1. Extraction efficiencies (%) of cholesterol and stigmasterol from mixed cultures.

Sterol	Aerobic Mixed Culture[a]		Sulfate-reducing Mixed Culture[a]	
	1 Extraction	3 Extractions	1 Extraction	3 Extractions
Cholesterol	95.8 ± 3.3[a]	98.3 ± 3.6	98.0 ± 2.7	100.5 ± 3.6
Stigmasterol	95.9 ± 3.6	98.5 ± 4.1	95.1 ± 2.6	97.8 ± 3.6

[a] Mean ± standard deviation ($\eta = 3$)

3.2.3. Trace Level Extraction and Sample Preparation

Samples with expected phytosterol concentrations below the method detection limit were analyzed using liquid-liquid extraction with iso-octane. Samples (200-500 mL) were poured into a glass graduated cylinder to record sample volumes and then transferred into 1 L glass bottle. The graduated cylinder was rinsed with a small volume of DI water which was then added to the sample. Sample pH was adjusted to >12 using

10 N NaOH solution, and cholesterol (8 g/L in ethanol) was spiked to a final concentration of 1 mg cholesterol/L to serve as a surrogate standard. As the samples were mixed rapidly on a magnetic stir plate, 100 mL of iso-octane was added to the bottle. The vigorous mixing of the sample with iso-octane was continued for at least 16 h while the bottle was sealed with a Teflon-lined plastic cap. After mixing, the contents of the bottle were transferred quantitatively to a 1000 mL separatory funnel, left quiescent for 2 h and the water layer was removed. The remaining extract was dried using anhydrous sodium sulfate and evaporated to dryness under a gentle stream of helium. The sample was reconstituted with 1 mL of iso-octane and transferred to a 2 mL autosampler vial for GC-FID/MS analysis. All cylinders, bottles and separatory funnels were washed with iso-octane prior to use.

3.2.4. GC-FID Quantification of Phytosterols

Quantification of phytosterols and cholesterol was performed using an Agilent 7890 gas chromatograph with FID and MSD. Separation of analytes was accomplished using a Zebron ZB-5HT, 0.25 mm ID column which terminated in a Dean's Switch for simultaneous collection of FID and MS data. The GC inlet was operated at 325°C and 23.4 psi using a splitless MS-certified inlet liner. Helium was used as carrier gas with a flow rate of 2.4 mL/min. The injection volume was 1 µL. Initial oven temperature was 90°C, which was held for one minute. The oven was ramped to 250°C at 20°C/min, held there for one minute, ramped to 310°C and held there for two minutes before termination of the run.

GC-MS was tested for use in quantifying sterols during the initial stages of method development. A modified EPA Method 1698 was used (USEPA, 2007). The GC

inlet and temperature ramping profiles as well as the hardware were the same as described above. The EI-MSD was operated in SIM mode and derivatization with N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) was used to increase sensitivity to target analytes, as follows. Samples were first extracted with methylene chloride. Derivatization with a silylation reagent requires removal of water and alcohols in addition to heating of the sample to increase reaction rate. Water was removed by passing the sample extract through a column of anhydrous sodium sulfate, and alcohol was removed by evaporating the extract to dryness followed by reconstitution with hexane. Several heating methods were reported in the literature, including by microwave and dry block heater (Bowden et al., 2009a; Shareef et al., 2006). Heating of the extract by microwave (maximum power level for 1 - 10 min) was attempted but produced inconsistent results, although the validity of that assertion is questionable as explained below. Heating in a dry block heater at 70°C for 45 min was reported to be the most efficient method for derivatization with MSTFA (Bowden et al., 2009b) and was used for the remaining phytosterol analysis method testing in this study. After the derivatized sample extract was allowed to cool, dichlorodiphenyldichloroethylene (DDE) was spiked as internal standard before GC-MS analysis. Table 3.2 shows the SIM ions used to identify each sterol. Reproducible analytical results were not obtained while using GC-MS to quantify phytosterols despite repeated trials over several months with the described method and variations omitting the derivatization step. Despite literature references (USEPA, 2007; Bowden et al., 2009b) to an increased response factor for the derivatized sterols over the underivatized forms, the detection limit was not found to differ for the derivatized and underivatized forms. Use of the MS for quantification also required frequent tuning of

the MS and subsequent recalibration of the sterol responses after each tuning. Due to the problems posed by analysis with MS, FID response was investigated for use in quantifying sterols. The detection limit using FID was similar to that obtained using MS, but FID analyses resulted in smaller relative standard deviations for standards and required less sample preparation. Eventually, MS quantification of sterols was abandoned in favor of FID.

Table 3.2. SIM ions used for GC-MS quantification of sterols.

Sterol	**SIM Ions, m/z**
Cholesterol-tms[a]	129, 329, 386, 458
Stigmasterol-tms	83, 129, 255, 484
β-sitosterol-tms	129, 357, 396, 486

[a] tms denotes trimethylsilyl derivative

FID analyses were performed on the above described equipment with identical instrument configuration and setting to those described above. FID heater temperature was 250°C. H_2 flow rate was 30 mL/min and air flow rate was 400 mL/min. Helium was used as the FID makeup gas with a constant column + makeup flow rate of 25 mL/min. Figure 3.1 shows a sample chromatogram with FID signal illustrating the analyte elution order and retention time. Calibration curves for cholesterol and stigmasterol are shown in Figure 3.2. Response factors for stigmasterol and cholesterol were 649153 and 608949, respectively. For the purposes of quantification, the response factor for each of the target sterols was assumed to be 650,000/mg/L. This was done for analytical convenience and

because cost and lack of availability prevented the purchase of suitable standards of β-sitosterol and campesterol. It is believed that this was a suitable method based on comparison of the certificate of analysis of the purchased phytosterol mixture to the results obtained by analysis using the above described method. The comparison of the phytosterol mixture analyses is shown in Table 3.3. The method detection limit for individual sterols was 50 µg/L when a sample:solvent ratio of 2:1 was used.

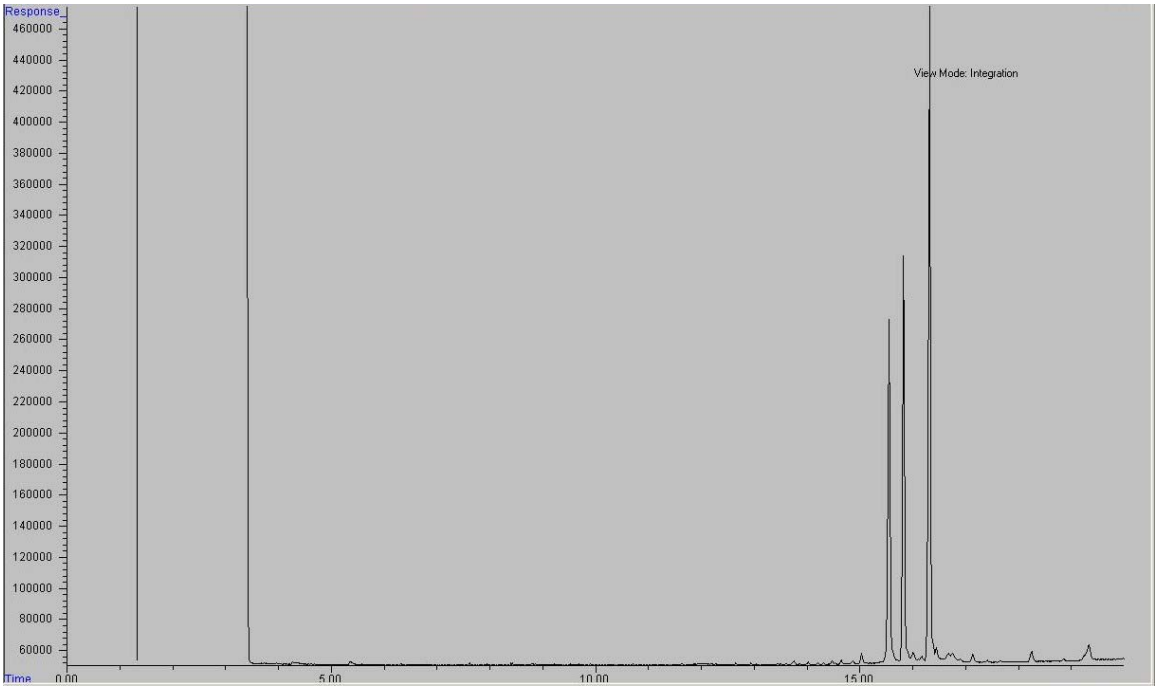


Figure 3.1. FID chromatogram of phytosterol mixture showing elution order and retention times for campesterol, stigmasterol and β-sitosterol at 1.25, 1.29 and 2.04 mg/L, respectively.

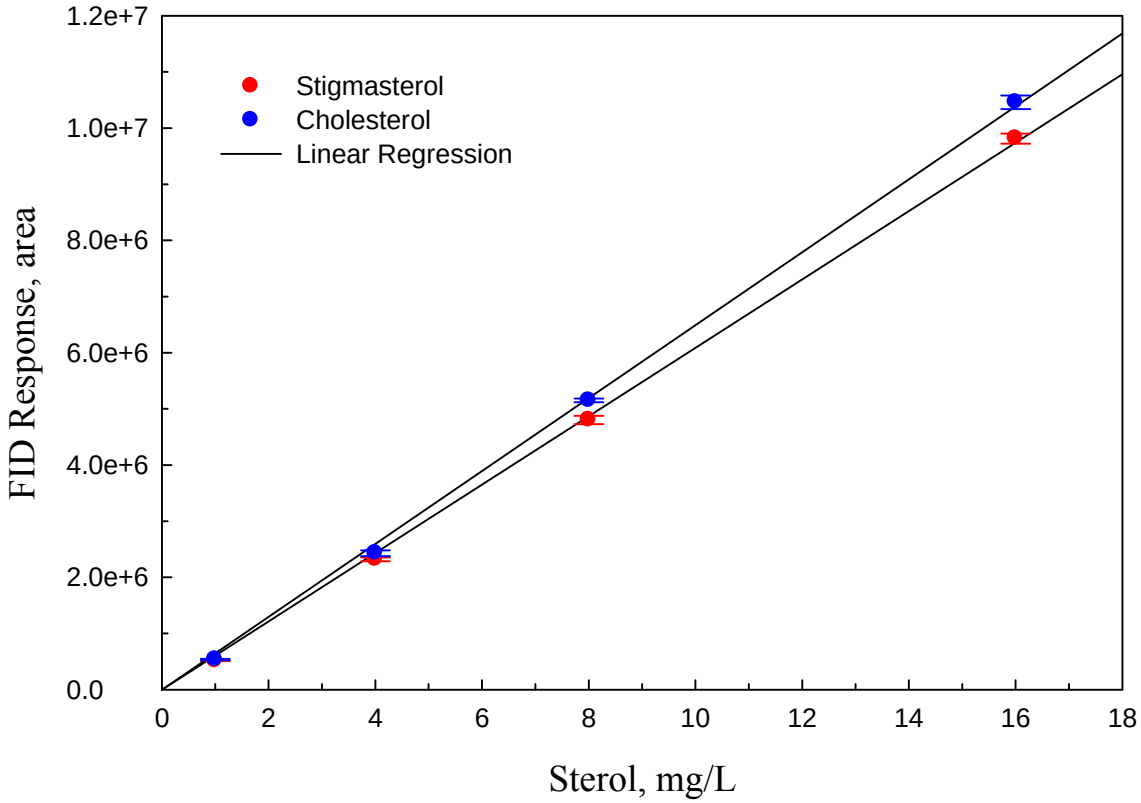


Figure 3.2. Calibration curves for cholesterol and stigmasterol. The response factors for cholesterol and stigmasterol are 649153/mg/L (r^2 0.989) and 608949/mg/L (r^2 0.997), respectively.

Table 3.3. Analyses of phytosterol mixture purchased from PureBulk.com.

Phytosterol	**Certificate of Analysis**	**Analysis by GC-FID**
Campesterol	23.0%	25.2%
Stigmasterol	26.0%	25.4%
β-sitosterol	40.0%	41.3%

3.2.5. GC-MS Identification of Phytosterols

Identification of sterols and other analytes was performed using an Agilent 7890 gas chromatograph with a 5975C inert XL EI-MSD. Separation of analytes was accomplished using a Zebron ZB-5HT, 0.25 mm ID column which terminated in a Dean's Switch for simultaneous collection of FID and MS data. The MSD was set to scan within the range of m/z 50-500 during the run after a solvent delay of 5 min. The MS Source and MS Quad were set to 230°C and 150°C, respectively. The ion polarity was positive. MS spectra were compared to purchased standards and the NIST library for identification of analytes. Figure 3.3 shows an example MS spectrum for β-sitosterol obtained using this method compared with a NIST library spectrum for β-sitosterol. GC parameters were as described in section 3.2.4, above.

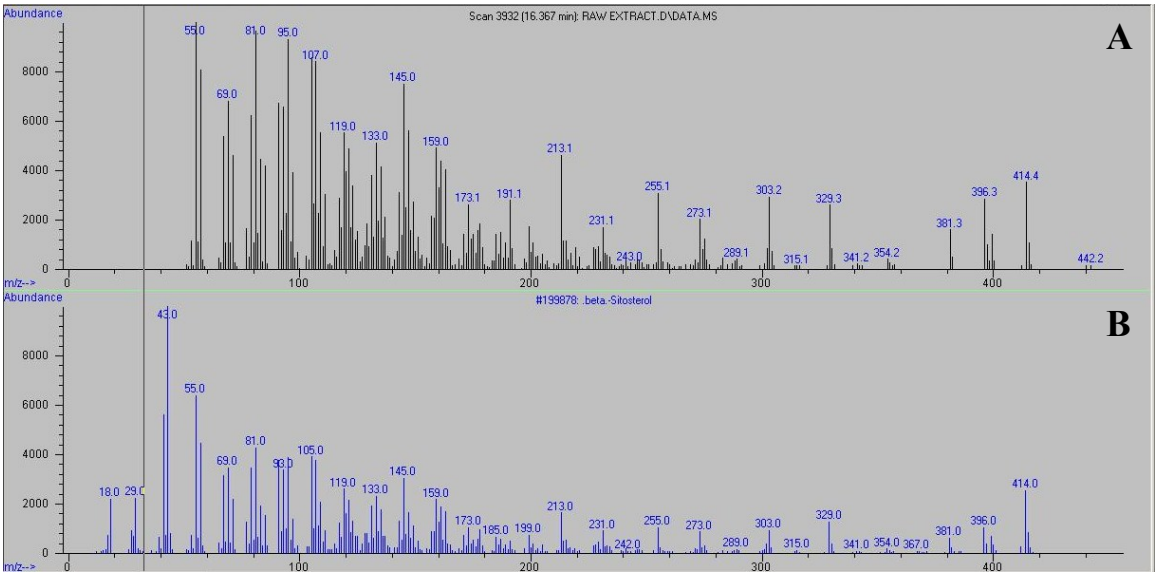


Figure 3.3. MS spectra of β-sitosterol for (A) the purchased phytosterol mixture and (B) the NIST library.

CHAPTER 4

AQUEOUS SOLUBILITY AND PHASE DISTRIBUTION

4.1. Introduction

Phytosterols are known endocrine disrupting compounds that can be found in many wastewater streams. They are natural components of eukaryotic cell membranes and are ubiquitous in the environment. Yet, important physical properties, such as aqueous solubility and partition coefficients, are unknown. Because of their endocrine disrupting potential, the ability to remove phytosterols from waste streams is of interest. The objective of this research was to assess the biotransformation potential of phytosterols in biological treatment systems. Biotransformation of phytosterols in biological treatment systems can reduce the discharge levels of these problematic compounds. To better understand phytosterol biotransformation potential, processes affecting their bioavailability have also been investigated.

A property that has been unexplored and plays an intricate role in the transport of phytosterols within biological systems is aqueous solubility. The aqueous solubility of cholesterol and of a phytosterol mixture was investigated using an apparatus which continuously circulates DI water from a reservoir through a column packed with sterol loaded sand. The only published value of aqueous solubility for a sterol is for cholesterol; thus, cholesterol was chosen as a model sterol for this experiment for comparison purposes. Phytosterols are usually present as a mixture rather than alone so a phytosterol mixture was chosen for investigation.

Solid-liquid partitioning affects the bioavailability of phytosterols within biological treatment systems. The ASBs used by pulp mills consist of an aerated surface zone and anaerobic sediments. Aeration is not uniform throughout the system, but rather is tapered, with aeration being concentrated near the wastewater inflow and reduced near the wastewater outflow to remove COD and allow solids to settle. Phytosterols, due to their hydrophobic nature, enter the biological treatment system adsorbed to wastewater solids, colloids, or dissolved organic matter. The wastewater then encounters the aerobic zone of the ASB. The phytosterols can desorb or remain adsorbed to wastewater solids or dissolved organic matter, which can affect their bioavailability. Desorbed phytosterols can then adsorb to aerobic biomass in the upper water zone. When they encounter quiescent conditions, both aerobic biomass and wastewater solids settle, transporting adsorbed phytosterols into the bottom sediment, where anaerobic processes, especially sulfate reduction and methanogenesis, take place. To evaluate these processes, batch adsorption and desorption assays were performed with model sterols, aerobic biomass and wastewater solids. Because pulp mill wastewater pH can vary, the effect of pH on stigmasterol adsorption and desorption to wastewater solids was investigated. Biological treatment systems typically function within a narrow pH range near neutral. For this reason, adsorption assays utilizing aerobic biomass were only performed at pH 7.

4.2. Materials and Methods

4.2.1. Target Compounds

The aqueous solubility of a single standard was investigated using cholesterol (≥ 99%) purchased from Sigma Aldrich. The aqueous solubility of phytosterols present as a

mixture was investigated using a phytosterol mixture purchased from PureBulk.com described in section 3.2.1, above.

4.2.2. Characterization of Pulp Mill Wastewater

Wastewater was collected twice during 2011from a southeastern US pulp and paper mill producing newsprint by the Kraft process from a combination of hardwood and softwood. In February 2011, approximately 100 gallons of untreated wastewater, 5 gallons of ASB mixed liquor, and 5 gallons of ASB sediment were collected for use in establishing and maintaining mixed cultures. The samples were stored without pH adjustment at 4°C until use. In October 2011, approximately 150 gallons of untreated wastewater was collected from the same southeast US mill.

The untreated wastewater was used to perform solid-liquid partitioning experiments assessing the partitioning of cholesterol and stigmasterol to pulp mill wastewater solids. Before use, the wastewater was analyzed for pH, ionic strength, TSS/VSS, tCOD/sCOD, total and soluble phenolics, total and soluble carbohydrates, total carbon, dissolved organic carbon, nitrate, ammonia, chloride, phosphate, sulfate and total and soluble phytosterols.

4.2.3. Aqueous Solubility Assay

The aqueous solubility of sterols, present alone or as a mixture, was investigated by measuring the sterol concentration in DI water after it had been continuously circulated for at least 4 weeks through a packed sand column loaded with sterol. The apparatus consisted of a water reservoir, a pump for circulating the water, a packed column, and copper lines extending from the reservoir to the pump, from the pump to the

base of the packed column and from the top of the packed column to the reservoir. Figure 4.1 shows the arrangement of the apparatus.

The column was prepared as follows: cholesterol or the phytosterol mixture were dissolved in methylene chloride to a concentration of 6 g total sterols/L to prepare two stock solutions. The sand used in the column was cleaned with methylene chloride and stored at 105°C before use. Sand (10 mL) was added to a 150 mL Erlenmeyer flask and 2 mL of the selected sterol stock solution was uniformly applied to the sand using a Pasteur pipette while mixing the sand sample. The methylene chloride in the stock solution was evaporated using a gentle stream of helium leaving a film of sterol on the sand particles. The copper line from the outlet of the circulating pump was inserted through a rubber stopper and extended to the base of a 25 mL culture tube. Clean sand (3 mL) was added to the culture tube followed by the sterols-loaded sand and then an additional 3 mL of clean sand. The culture tube was then sealed with the rubber stopper, and the copper line used to return the water to the reservoir was inserted through the stopper to a height of approximately 2 cm above the sand. The column was soaked with clean DI water for 2 hours before beginning circulation and the first 100 mL of water exiting the column was discarded to remove any possibly dislodged sterol particles. The column was operated with the water level above the level of sand but below the rubber stopper in order for the reservoir return line to remain submerged but the water not come into contact with the rubber stopper.

DI water was continuously circulated through the column using a positive displacement FYI Lab Pump at a flow rate of 2.1 mL/min for approximately 4 weeks before sampling. Water was sampled through a glass port at the base of the water

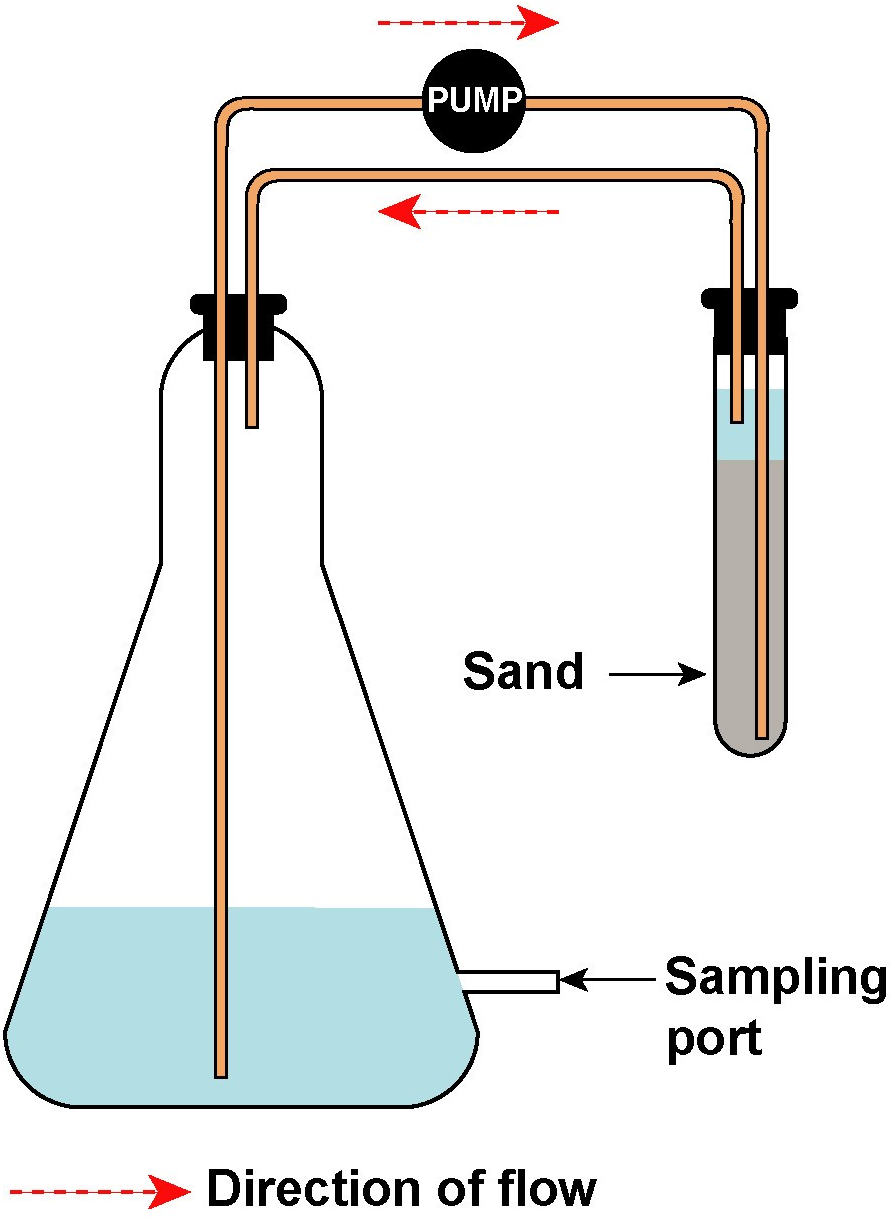


Figure 4.1. The apparatus used for the assessment of sterol aqueous solubility.

reservoir controlled by a Teflon stopcock. Samples were collected by allowing the water in the reservoir to freely flow through the port into a 250 mL separatory funnel. The sample volume was determined by weight using the difference between the weight of the separatory funnel containing the sample and the tare weight of the separatory funnel. The experiment was performed at 22°C. The water samples were extracted with 20 mL of iso-octane by shaking vigorously for 20 minutes, and the extract was dried with anhydrous sodium sulfate and evaporated to dryness by a gentle stream of helium. The residue was reconstituted with 1 mL of iso-octane, transferred to an autosampler vial with a glass Pasteur pipette and analyzed by GC-FID to quantify sterols. The concentration factor was calculated as the volume of the water sample divided by the reconstituted volume of the solvent extract, and the concentration determined by GC-FID was adjusted accordingly.

4.2.4. Solids Partitioning Assays

This section summarizes the analyses performed to assess the solid-liquid partitioning of individual sterols in the presence of aerobic mixed culture biomass and pulp mill wastewater solids.

4.2.4.1. Aerobic Culture Biomass Partitioning Assays

Aerobic culture biomass partitioning assays were carried out in 30 mL Kimble Kimax centrifuge tubes with PTFE lined caps purchased from Sigma Aldrich. Aerobic mixed culture (20 mL) was added to each tube and either cholesterol or stigmasterol, dissolved in ethanol, was added to the desired final concentration. Stock solutions of cholesterol and stigmasterol dissolved in ethanol contained 8 g/L and 4 g/L sterol, respectively. Sodium azide (200 mg/L) was added to the aerobic culture biomass to

inhibit all biological activity during the partitioning assays. The tubes were vortexed for 1 minute and placed on an orbital shaker (190 rpm) for 24 hours to equilibrate. The equilibration time was determined after initial testing to determine time to equilibrium. After equilibration, the tubes were centrifuged at 5000 rpm for 30 minutes. The supernatant was removed, extracted with iso-octane, and then analyzed by GC-FID for sterol quantitation.

All glassware was rinsed with iso-octane prior to use. Experiments were performed at 22°C and pH 7. Stock solutions were transferred using a glass microsyringe with a stainless steel needle. Supernatant volumes were determined using a 20 mL graduated glass cylinder. Sterol concentration in the solid phase was calculated according to Equation 4.1:

(Equation 4.1)
$$q_e = \frac{M_T - (C_e \times V_{sample})}{VSS_{sample} \times V_{sample}}$$

In Equation 4.1, q_e is the equilibrium sterol concentration on the solids (µg sterol/mg VSS), M_T is the total mass of sterol in the system (µg), C_e is the equilibrium sterol concentration in the liquid phase (µg sterol/L), V_{sample} is the sample volume (L), and VSS_{sample} is the VSS concentration of the sample (mg VSS/L).

The equilibrium liquid phase concentrations were plotted against the solid phase concentrations, and the resulting data points were fitted to the Freundlich isotherm equation, Equation 4.2, below:

(Equation 4.2)
$$q_e = K_f C_e^{\,n}$$

In Equation 4.2, q_e is the equilibrium sterol concentration on the solids (µg sterol/mg VSS), C_e is the equilibrium sterol concentration in the liquid phase (µg sterol/L), K_f is the capacity factor ((µg/mg VSS)(L/µg)n), and n is the Freundlich exponent. The two adsorption constants (K_f and n) were determined by non-linear regression based on equation 4.2, using SigmaPlot.

4.2.4.2. Wastewater Solids Partitioning Assays

Sterol adsorption assays with cholesterol and stigmasterol using the pulp mill wastewater were performed as described in section 4.2.3.1, above, except, separate assays were performed at pH 7 and pH 9. Freundlich constants were estimated according to Equation 4.2.

Desorption assays were also performed for pulp mill wastewater solids. For the desorption assays, 24 hour initial equilibrations were performed in the manner described in section 4.2.3.1, above, at pH 7 and initial stigmasterol concentration of 6.8 mg/L. After centrifugation, the supernatant was decanted and extracted to determine the liquid phase stigmasterol concentration. The initial mass of stigmasterol adsorbed to the wastewater solids was calculated according to the Equation 4.3, below, where $M_{adsorbed}$ is the mass of sterol adsorbed to the solids.

(Equation 4.3) $$M_{adsorbed} = M_T - (C_e \times V_{sample})$$

After initial equilibration, the wastewater solids were desorbed in 8 successive desorption steps, each lasting 24 hours and at desorption pH 5, 7 and 9. Desorption steps were performed as follows: tubes were removed from the orbital shaker and centrifuged at 5000 rpm for 30 min at 20°C. The tubes were weighed and the supernatant was decanted into a 25 mL culture tube for extraction and analysis for stigmasterol

concentration by GC-FID. The tubes were reweighed, and the weight difference was used to calculate the volume of supernatant removed assuming a density of water of 0.998 g/mL at 22°C. The volume of supernatant that was removed was replaced with DI water containing phosphate buffer (1.5 mM KH_2PO_4 and K_2HPO_4), which had been adjusted to the desired pH with either HCl or NaOH. Then, the tubes were placed on the orbital shaker to mix for an additional 24 hours. The adsorbed mass of stigmasterol remaining after each desorption step was calculated according to Equation 4.4, below:

(Equation 4.4) $$M_n = M_{n-1} - \left\{C_n \times V_s - \left[C_{n-1} \times (V_s\text{-}V_r)\right]\right\}$$

In Equation 4.4, M_n is the mass of sterol remaining after the n desorption step, M_{n-1} is the mass of sterol present before the n desorption step, C_n is the liquid phase concentration, C_{n-1} is the liquid phase concentration from the previous desorption step, V_s is the total sample volume for the n desorption step and V_r is the volume of liquid that was left after the removal of supernatant during the previous desorption step.

4.3. Results and Discussion

4.3.1. Characteristics of Pulp Mill Wastewater

The characteristics of the pulp mill wastewaters collected in February and October of 2011 are given in Table 4.1. The ratio of the parameter value in the October wastewater over the parameter value in the February wastewater is also given, for comparison purposes, in Table 4.1. The pH of the wastewater did not change significantly between February and October, and remained near pH 10. Pulp mill wastewater is an agglomeration of multiple wastewater streams originating at multiple points within the pulp mill. Some of these wastewater streams have neutral or acidic pH

values depending on the process type being employed. The pH of the wastewater stream entering the biological treatment system is therefore highly variable depending on the relative flow rates of the different wastewater streams exiting the mill, which can change in magnitude over time. With the exception of pH, parameter concentrations were higher in the October wastewater than the February wastewater. The simultaneous increase in concentration of so many wastewater components would suggest that the mill was using less water during October than February; however, no change in mill operation was reported by the mill management. TSS was more than twice VSS in both wastewaters indicating that the wastewaters contained a high percentage of inorganic solids, 59 and 53 % of TSS for the February and October wastewaters, respectively. Soluble COD comprised 31 and 38 % of total COD in the February and October wastewaters, respectively. Soluble phenolics comprised 80 and 72 % of total phenolics in the February and October wastewaters, respectively. Soluble carbohydrates comprised 4 and 9 % of total carbohydrates in the February and October wastewaters, respectively. The source of phenolics and carbohydrates in the wastewater is lignin and cellulose/hemicellulose, respectively. Phenolic compounds are liberated from wood fibers and dissolved into solution during the pulping process, which accounts for the high percentage of soluble phenolics. Carbohydrates in the wastewater are dominantly in the form of insoluble cellulose fibers, accounting for the low dissolved carbohydrate percentage. DOC comprised 22 and 16 % of TC in the February and October wastewaters, respectively. Nitrate was only detected in the February wastewater and ammonia was not detected in either wastewater. The concentration of nitrate in the February wastewater was very low. Phosphate was only detected in the October wastewater. Total phytosterol concentrations

Table 4.1. Characteristics of pulp and paper mill wastewaters collected in February and October 2011.

Parameter	February 2011	October 2011	Ratio[c]
pH	10.0	10.5	---
Ionic Strength, mol/L	0.014	0.033	1:2.4
TSS, g/L	0.66 ± 0.01[a]	2.30 ± 0.04	1:3.5
VSS, g/L	0.27 ± 0.01	1.09 ± 0.04	1:4
Total COD, mg/L	903 ± 102	2356 ± 63	1:2.6
Soluble COD, mg/L	279 ± 20	900 ± 8	1:3.2
Total Phenolics, mg/L gallic acid equivalents	32.9 ± 1.3	148.4 ± 4.0	1:4.5
Soluble Phenolics, mg/L gallic acid equivalents	26.5 ± 1.8	106.6 ± 9.3	1:4.1
Total Carbohydrates, mg/L as glucose	250 ± 24	768 ± 80	1:3
Soluble Carbohydrates, mg/L as glucose	9.7 ± 3.3	68 ± 22	1:6.8
TC, mg/L	579 ± 50	1510 ± 80	1:1.3
DOC, mg/L	129 ± 2	250 ± 13	1:1.9
Nitrate, mg N/L	0.24 ± 0.04	ND	---
Ammonia, mg N/L	ND[b]	ND	---
Chloride, mg/L	18.8 ± 3.3	84.2 ± 3.4	1:4.5
Phosphate, mg P/L	ND	20.7 ± 3.5	---
Sulfate, mg S/L	43.6 ± 6.8	98.7 ± 4.2	1:2.3
Total β-sitosterol, µg/L	69.2	305.3 ± 7.5	1:4.4
Soluble β-sitosterol, µg/L	22.8	96.0 ± 4.6	1:4.2
Total Stigmasterol, µg/L	22.6	52.7 ± 2.3	1:2.3
Soluble Stigmasterol, µg/L	5.0	6.6 ± 0.3	1:1.3
Total Campesterol, µg/L	9.3	43.9 ± 2.4	1:4.9
Soluble Campesterol, µg/L	2.1	10.5 ± 1.0	1:5

[a] Mean ± standard deviation ($\eta = 3$); [b] ND, not detected; [c] Ratio = Value in February 2011 over value in October 2011

were at least 3 times higher than soluble phytosterol concentrations indicating that phytosterols in the wastewater were predominantly associated with the wastewater solids. It should be noted that the method described for extracting trace levels of sterols in Chapter 3 section 3.2.3, was not used to quantify phytosterols in the February wastewater; thus, actual phytosterol concentrations in the February wastewater may be higher than indicated in Table 4.1.

4.3.2. Assessment of Aqueous Solubility of Phytosterols

The cholesterol aqueous solubility was measured after 4 weeks of continuous water circulation and again after another week (i.e., 5 weeks total) to confirm that the solubility limit had been reached. At the first sampling, the measured cholesterol concentration was 2.24 ± 0.38 μg/L (mean ± std. dev., $\eta = 2$). The second sampling resulted in a measured cholesterol concentration of 6.34 ± 5.19 μg/L (mean ± std. dev., $\eta = 3$).

The high relative standard deviation during the second sampling period, 82%, does not permit a definitive statement as to the value for the aqueous solubility of cholesterol. However, it can be safely assumed that the aqueous solubility of cholesterol is in the single digit μg/L range.. This is consistent with software estimates of the aqueous solubility of cholesterol reported as 3.6 and 3.3 μg/L by WATERNT (EPISuite) and ACD/Labs software, respectively. An experimental value of 95 μg/L is reported in the literature for the aqueous solubility of cholesterol at 30°C (VCCLAB); however this reported value, far exceeds the concentrations achieved in the present study and those based on software estimates.

The aqueous solubility of the phytosterol mixture was assessed after 2, 4 and 8 weeks of continuous water circulation. After 2 and 4 weeks, sterols could not be detected at water:solvent concentration factors of 60:1 and 88:1, respectively. A concentration factor of 88:1 permits the detection of individual sterol concentrations as low as 0.57 µg/L. A sample volume of 250 mL was taken after 8 weeks to achieve a concentration factor of 250:1. This concentration factor allows the detection of individual sterol concentrations as low as 0.2 µg/L. After 8 weeks, the aqueous concentration of campesterol, stigmasterol and β-sitosterol was 0.2, 0.2 and 0.3 µg/L, respectively.

Phytosterols present as a mixture were soluble to a lesser extent than cholesterol present alone. The individual aqueous phytosterol concentrations measured in the present study are an order of magnitude lower than the measured values for cholesterol. The aggregate concentration of the three phytosterols (0.7 µg/L) was also less than the concentration of cholesterol at saturation. No experimental values for aqueous solubility of phytosterols are available for comparison; however, estimates of individual phytosterol concentrations range from 0.5 µg/L for β-sitosterol (WATERNT) to 2.0 µg/L for campesterol (ACD/Labs). Thus, the measured values in the present study are within the same order of magnitude of reported software estimates.

4.3.3. Assessment of Solids Partitioning of Phytosterols

4.3.3.1. Aerobic Culture Biomass Adsorption Assays

Cholesterol adsorption to aerobic mixed culture biomass was investigated at pH 7 and 22°C for total cholesterol concentrations of 1, 4, 6, 8, 10, 16 and 24 mg/L. Exceeding 10 mg/L cholesterol resulted in cholesterol crystals forming at the air/water

interface during centrifugation, interpreted as having exceeded the capacity of the water to dissolve the total mass of cholesterol added. Therefore, samples at 16 and 24 mg/L were excluded from the adsorption isotherm.

Stigmasterol adsorption to aerobic mixed culture biomass was investigated at pH 7 and 22°C for total stigmasterol concentrations of 1, 2, 3, 4, 6 and 8 mg/L. Exceeding 4 mg/L stigmasterol resulted in the formation of stigmasterol crystals; thus, samples at 6 and 8 mg/L were discarded. Figure 4.2 shows the adsorption isotherms for aerobic culture biomass at pH 7 and 22°C for cholesterol and stigmasterol.

Freundlich coefficients (K_f, capacity factor/sorption affinity and n, exponent) for the cholesterol and stigmasterol isotherms are shown in Table 4.2. The r^2 values indicate that the Freundlich isotherm model represents the sterols adsorption behavior well. As indicated by their low K_f values, both sterols have a relatively low affinity for aerobic culture biomass; however, cholesterol has about a two-fold higher affinity than stigmasterol. The low biomass affinity of the sterols is not surprising. The surface of biomass is hydrophilic and sterols are classified as very hydrophobic ($\log K_{ow} > 7$). Although a sample size of two compounds makes a definitive correlation impossible,

Table 4.2. Freundlich constants estimated for cholesterol and stigmasterol isotherms with aerobic culture biomass at pH 7 and 22°C.

Sterol	**K_f (µg/mg VSS)(L/µg)*n**	**n**	**r^2**
Cholesterol	0.698 ± 0.199[a]	0.676 ± 0.041[a]	0.996
Stigmasterol	0.334 ± 0.142	0.463 ± 0.075	0.974

[a] Mean ± standard error

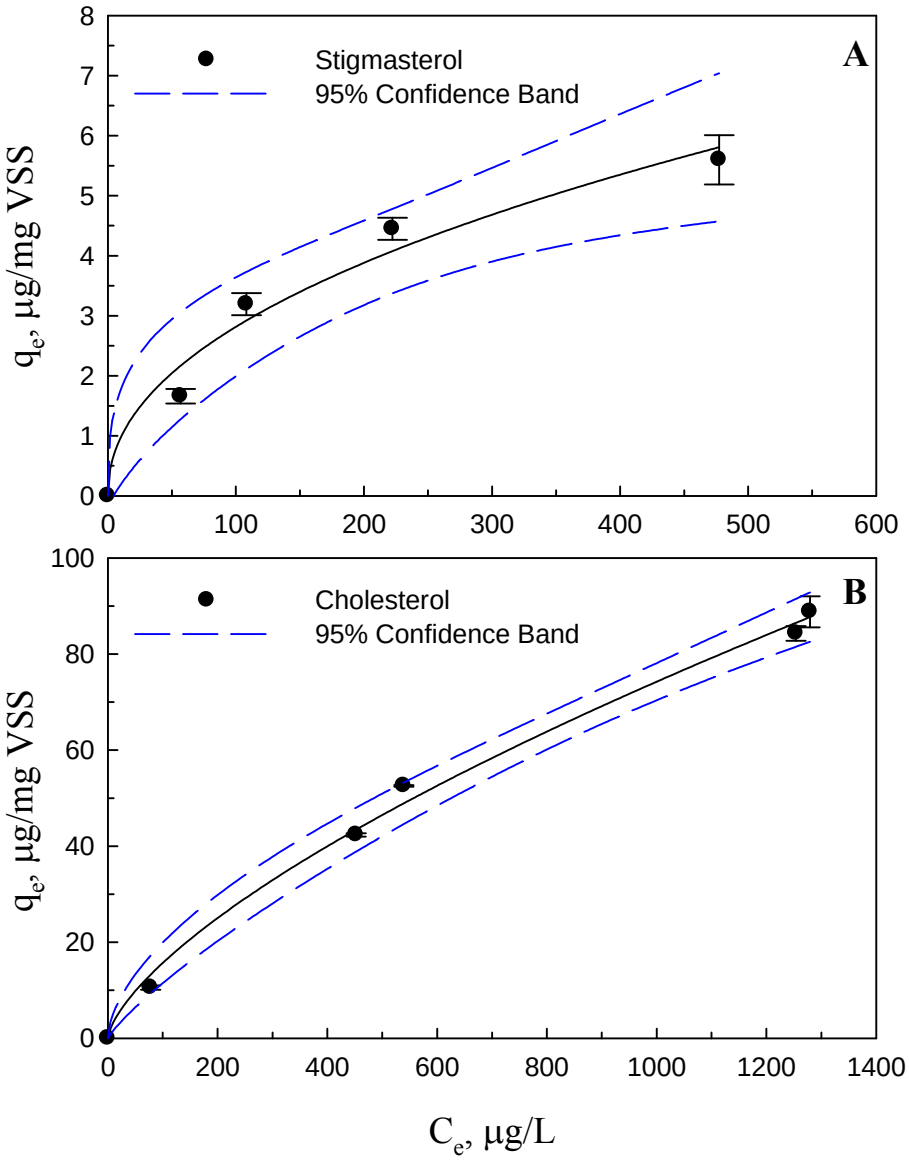


Figure 4.2. Adsorption isotherms of (A) stigmasterol and (B) cholesterol using aerobic culture biomass (Tests performed at pH 7 and 22°C) (Error bars represent one standard deviation of the mean).

the observed relative biomass affinity coefficients indicate that increased branching of the sterol side chain reduces biomass affinity.

The n values for both cholesterol and stigmasterol are below 1, which indicates that as the total sterol concentration increases, adsorption becomes less favorable. This often occurs when easily accessible binding sites are occupied at low sorbate concentrations leaving less accessible binding sites (Schwarzenbach et al., 2003). The liquid phase concentrations observed during the assays were well above the observed and estimated solubility limits for cholesterol and stigmasterol, respectively. Such high concentrations are attributed to the adsorption of the sterols to dissolved organic matter present in the samples, which remains suspended after centrifugation and increases the apparent aqueous solubility of the sterols.

4.3.3.2. Wastewater Solids Adsorption Assays

Cholesterol adsorption to pulp mill wastewater solids was investigated at pH 10, which is the pH value of the specific wastewater used in this study, and 22°C for total cholesterol concentrations of 1, 4, 6, 8, 10 and 12 mg/L. Stigmasterol adsorption to pulp mill wastewater solids was investigated at pH 7 and pH 10 and total stigmasterol concentrations of 1, 2, 3 and 4 mg/L. The effect of pH on the adsorption of sterols on pulp mill wastewater solids is of interest because of the range of pH that can exist in pulp mill biological treatment systems. The pH of the pulp mill wastewater used for these assays was 10. To investigate the effect of lower pH values on adsorption to wastewater solids, assays were performed at pH 7 and pH 4; however, adjusting the wastewater pH to 4 resulted in breakdown (i.e., destabilization) of the wastewater solids and prevented the

recovery of a solids-free supernatant. The results of this assay were highly erratic due to the presence of solids in the supernatant and were discarded.

The adsorption isotherms resulting from the successful assays are displayed in Figure 4.3. Estimated values for the Freundlich constants derived from the data are given in Table 4.3. The r^2 values indicate that the Freundlich isotherm model represents the adsorption of sterols well. The affinity of both sterols for pulp mill wastewater solids was 2 orders of magnitude lower than the observed affinities of the sterols for aerobic biomass. It is not surprising that the affinity for wastewater solids is low. For all practical purposes, pulp mill wastewater solids consist of cellulose fibers. By the time the fibers have been processed in the pulp mill, the hydrophobic components of the wood have been removed leaving a hydrophilic surface. This is to encourage hydrogen bonding of the fibers to one another, resulting in stronger paper. Because of the hydrophobic nature of the sterols, their affinity for cellulose fibers would not be high.

The affinity of cholesterol for wastewater solids at pH 10 did not change with cholesterol concentration within the range investigated. Stigmasterol, however, displayed a slightly increased affinity for the wastewater solids as stigmasterol concentration

Table 4.3. Freundlich constants estimated for cholesterol isotherm at pH 10 and stigmasterol isotherms at pH 7 and pH 10 with pulp mill wastewater solids (Tests performed at 22°C).

Conditions	**K_f (µg/mg VSS)(L/µg)*n**	**n**	**r^2**
Stigmasterol, pH 7	0.0013 ± 0.0036	1.161 ± 0.447	0.904
Stigmasterol, pH 10	0.0007 ± 0.0007	1.275 ± 0.154	0.988
Cholesterol, pH 10	0.0059 ± 0.0085[a]	0.985 ± 0.169[a]	0.973

[a] Mean ± standard error

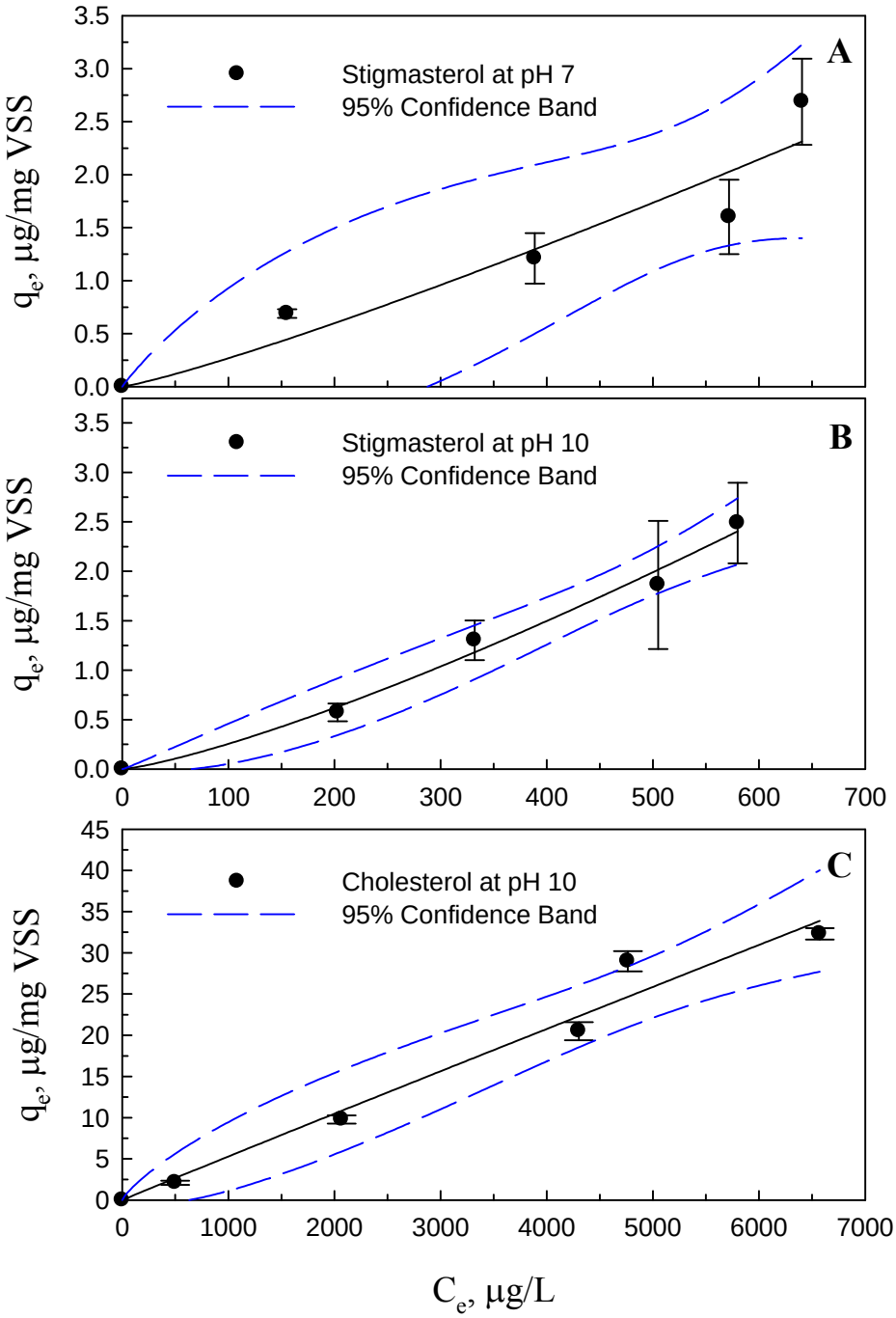


Figure 4.3. Adsorption isotherms of (A) Stigmasterol at pH 7, (B) Stigmasterol at pH 10, (C) Cholesterol at pH 10 using pulp mill wastewater solids (Tests performed at 22°C) (Error bars represent one standard deviation of the mean).

increased at both pH 10 and pH 7. It is noted that the liquid phase concentration observed during the wastewater adsorption assays is much higher than the liquid phase concentrations achieved during the adsorption assays with aerobic culture biomass. Such relatively high liquid phase concentrations are attributed to a higher concentration of dissolved organics in the wastewater. The wastewater used in these assays contained soluble COD of 900 ± 8 mg/L (mean ± std. dev.; n=3), whereas the aerobic culture contained soluble COD of 107 ± 4 mg/L (mean ± std. dev.; n=3) indicating a higher dissolved organic content in the wastewater.

4.3.3.3. Wastewater Solids Desorption Assays

Results for the wastewater solids desorption assays are shown in Figure 4.4 as a function of pH during desorption. For each pH tested, desorption followed a biphasic pattern with a rapid initial phase followed by a slower second phase. At pH 9, an increase in the initial phase desorption rate over the rate at lower pH values was observed. The observed rate was about 4 times greater than the rate at the lower pH values. The reason for this phenomenon could be a change in the cellulose surface charge or a change in the cellulose morphology. The net charge on the surface of a cellulose fiber can change with pH. Factors such as wood composition and pulping, bleaching and refining conditions affect the chemical and physical nature of the fiber surface; therefore, the magnitude and extent of the change is hard to predict and usually requires experimental testing to validate. An increase in pH can also cause a change in the macrostructure of the cellulosic fibers, causing fiber swelling and changes in the crystalline structure in what is known as the *mercerization process*.

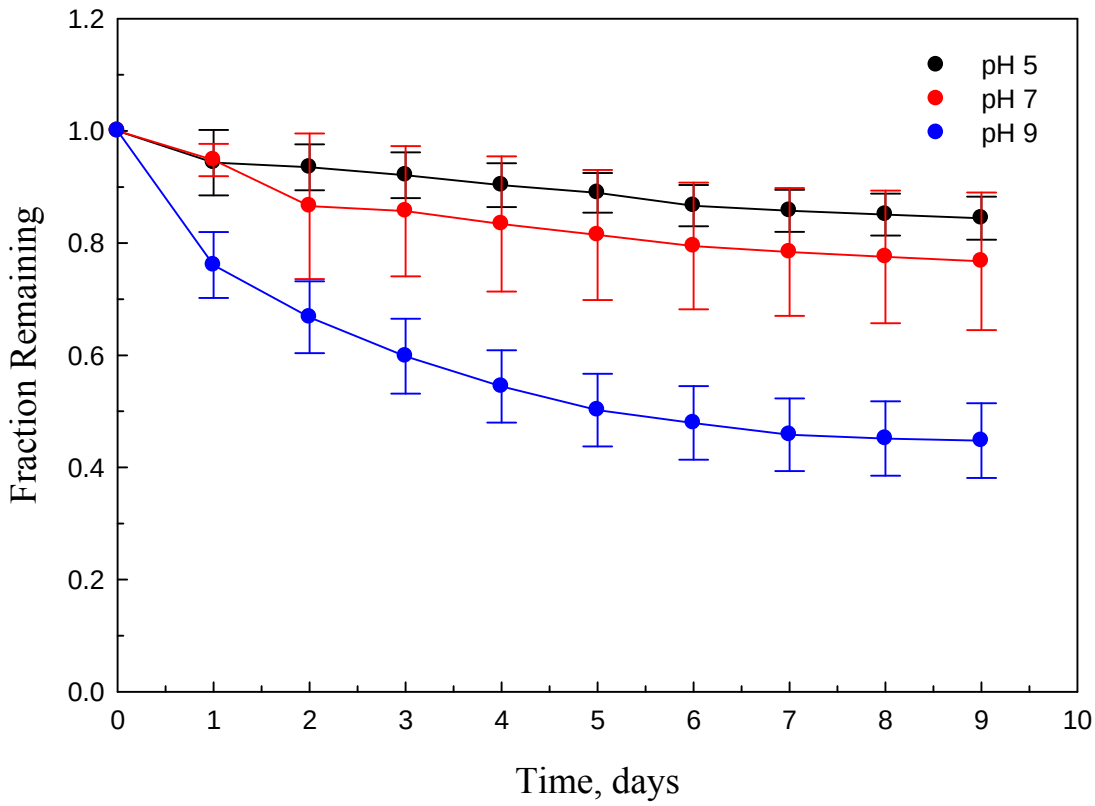


Figure 4.4. Solid-phase stigmasterol fraction remaining in pulp mill wastewater solids during sequential desorption steps performed at pH 5, 7 and 9 (Tests performed at 22°C) (Error bars represent one standard deviation of the mean).

After the rapid initial phase of desorption, desorption rate slowed until a steady-state was achieved at day 7. The steady-state desorption rate was not pH dependent, as the equivalent rates observed for the three assays from day 7 to 9 shows. The final extent of desorption after 9 successive desorption steps, each lasting for 1 day, was 16, 23 and 55 % at pH 5, 7 and 9, respectively. It should be noted that, because the assays were performed using centrifugation instead of filtration, a negligible loss of solids may have been unavoidable during decanting of the supernatant; however, because the desorption rate leveled off in each assay after 7 successive desorption steps, the observed decrease in solid-phase stigmasterol is believed to be due to a desorption of stigmasterol and not a loss of wastewater solids. If the losses of stigmasterol which were observed were due to a loss of solids, an equivalent or even increased rate of stigmasterol loss would be expected after 7 days from the breakdown of fibers as a result of continuous sample agitation.

4.4. Summary

Experiments were performed to assess the aqueous solubility and phase partitioning of select sterols. Cholesterol was found to have an intrinsic aqueous solubility in the single µg/L range. A more precise determination was not possible. The solubility of a mixture of three common phytosterols was also investigated. Phytosterol concentrations were measurable only at a concentration factor of 250:1. Dissolved phytosterol concentrations of 0.2, 0.2 and 0.3 µg/L were measured for campesterol, stigmasterol and β-sitosterol, respectively.

Solid-liquid partitioning assays were carried out with cholesterol and stigmasterol, aerobic culture biomass and pulp mill wastewater solids. Assays utilizing aerobic culture

biomass carried out at pH 7 showed that both sterols have a low affinity for the biomass, but cholesterol has a greater affinity than stigmasterol. Both sterols show a decreasing affinity for the culture biomass with increasing sterol concentration. Adsorption assays conducted with pulp mill wastewater solids were carried out at pH 7 for cholesterol and pH 5 and 7 for stigmasterol. The affinity for the wastewater solids was 2 orders of magnitude less than for the culture biomass for both sterols, regardless of pH. The affinity of stigmasterol for the wastewater solids was lower than that of cholesterol; the affinity did not decrease with increasing sterol concentration.

The desorption rate and extent of stigmasterol from pulp mill wastewater solids was investigated as a function of pH during desorption. Desorption followed a two-phase pattern, a fast initial phase followed by a slower second phase. The rate of the initial desorption phase was affected by pH. Assays performed at pH 5 and 7 had similar initial desorption rates while the assay performed at pH 9 had a higher initial rate. The slower second phase of desorption was equivalent for each of the assays, regardless of pH. The ultimate desorption extent after 9 successive desorption steps was 16, 23 and 55 % at pH 5, 7 and 9, respectively.

CHAPTER 5

BIOTRANSFORMATION POTENTIAL OF PHYTOSTEROLS UNDER VARIOUS REDOX CONDITIONS

5.1. Introduction

Very little research has been performed to assess the biotransformation potential of phytosterols within biological treatment systems. Aerobic biotransformation of phytosterols has been previously reported but deserves further scrutiny within the context of pulp mill wastewater treatment systems. Biotransformation of cholesterol by a limited number of species under nitrate-reducing conditions has been previously reported; however, biotransformation of phytoterols under nitrate-reducing conditions has not been investigated. Biotranformation of phytosterols under anaerobic conditions has not been investigated; however, cholesterol is reported to be recalcitrant under anaerobic conditions with the exception of limited conversion to cholestanol by fermentative bacteria.

The objective of the research reported in this chapter was to assess the biotransformation potential of phytosterols under aerobic, nitrate-reducing, sulfate-reducing and methanogenic conditions using bioenergetic calculations and biotransformation experiments. Cholesterol was used as a model sterol during preliminary experiments so that results could be compared to data already available in the literature.

5.2. Materials and Methods

5.2.1. Mixed Cultures

5.2.1.1. Aerobic and Nitrate-reducing Mixed Culture

An aerobic mixed microbial culture was developed for use in batch phytosterol biotransformation experiments. The inoculum for the culture was mixed liquor collected from a pulp mill ASB in the southeastern U. S. In order to closely simulate a representative pulp mill biological treatment environment, the culture was fed settled, untreated pulp mill wastewater as carbon and energy source. The culture was established from ASB mixed liquor (4 L). The mixed liquor characteristics are given in Table 5.1. Wastewater collected in February 2011, characteristics shown in Table 4.1, was used as feed and supplemented with $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ each to 60 mg/L, NH_4Cl to 60 mg N/L, phosphate buffer (pH 7.2; 1:1, KH_2PO_4:K_2HPO_4) to 30 mM and 1 mL of trace metals solution per liter (Table 5.2). Characteristics of the aerobic culture feed are given in Table 5.3. The wastewater collected in October 2011 was diluted 1:3 and supplemented with nutrients as described previously before being used as feed. Dilution of the wastewater was necessary to maintain continuity of the culture operating conditions. The raw wastewater was settled at 4°C and the solids were removed prior to the wastewater being used as feed. The wastewater was adjusted to pH 7 before use. Phosphate (1:1, KH_2PO_4:K_2HPO_4) was added in excess of the stoichiometric requirement to buffer pH. Ammonia (NH_4Cl) was added in excess of microbial growth requirements to stimulate ammonia oxidation to nitrate (i.e., nitrification). Nitrate, produced as a result of nitrification, was then removed by incorporation of a one day anoxic stage into the

Table 5.1. Characteristics of pulp mill ASB mixed liquor used to inoculate the aerobic stock culture and continuous-flow system.

Component	Concentration
pH	7.1 ± 0.1[a]
TSS, g/L	0.09 ± 0.01
VSS, g/L	0.06 ± 0.01
tCOD, mg/L	754 ± 78
sCOD, mg/L	317 ± 16
Chloride, mg Cl/L	96.5
Nitrate, mg N/L	0.9
Ammonia, mg N/L	ND[b]
Phosphate, mg P/L	73.5
Sulfate, mg S/L	143.9

[a]Mean ± std. dev., n = 3; [b] ND, not detected

Table 5.2. Composition of trace metals stock solution.

Chemical	Concentration
$ZnCl_2$	0.5 g/L
$MnCl_2.4H_2O$	0.3 g/L
H_3BO_3	3.0 g/L
$CoCl_2.6H_2O$	2.0 g/L
$CuCl_2.2H_2O$	0.1 g/L
$NiSO_4.6H_2O$	0.2 g/L
$Na_2MoO_4.2H_2O$	0.3 g/L

Table 5.3. Composition of aerobic culture feed.

Component	Concentration
pH	7.3 ± 0.2 [a]
Total COD, mg/L	351 ± 5
Soluble COD, mg/L	300 ± 3
$CaCl_2 \cdot 2H_2O$, mg/L	60
$MgCl_2 \cdot 6H_2O$, mg/L	60
$FeCl_2 \cdot 4H_2O$, mg/L	60
NH_4Cl, mg N/L	60
$NaNO_3$, mg N/L	ND[b]
Phosphate Buffer (1:1, KH_2PO_4:K2HPO4; pH 7.2), mM	30
Trace metals solution, mL/L	1

[a]Mean ± std. dev., $\eta = 3$; [b]ND, not detected

weekly operating cycle. This culture was therefore suitable for biotransformation experiments requiring aerobic, ammonia oxidizing or nitrate-reducing conditions. The culture was operated at a hydraulic retention time of 4.2 days and a solids retention time of 21 days.

The aerobic culture was fed untreated pulp mill wastewater which contained phytosterols. To assess the biodegradation of phytosterols by the aerobic stock culture during normal culture maintenance, phytosterol concentrations of the feed, culture liquid phase (i.e., settle culture supernatant) and culture mixed liquor were measured. The steady-state concentrations of the culture liquid phase and culture mixed liquor were estimated for comparison using the experimental Freundlich coefficients for stigmasterol measured as explained in Chapter 4, Section 4.3.2.1.

5.2.1.2. Sulfate-reducing Mixed Culture

A sulfate-reducing mixed microbial culture was developed for use in experiments investigating biotransformation of phytosterols under sulfate-reducing conditions. The

culture was developed from an ASB sediment inoculum and fed pulp mill wastewater in order to simulate a sulfate-reducing environment found in pulp mill ASB bottom sediments. The settled solids, removed from the untreated pulp mill wastewater when making the aerobic culture feed, were amended with nutrients (NH_4Cl to 120 mg N/L; phosphate buffer pH 7.2; 1:1, KH_2PO_4:K_2HPO_4 to 12 mM; Na_2SO_4 to 1479 mg S/L; anaerobic culture media to 100 mL media/L, Tables 5.4 and 5.5) and used as feed for the sulfate-reducing culture. Characteristics of the sulfate-reducing culture feed are given in Table 5.6. The culture was operated at a hydraulic retention time equal to the solids retention time of 21 days. Possible alternate carbon sources were investigated shortly

Table 5.4. Composition of anaerobic culture media.

Compound/Solution	**Concentration**
K_2HPO_4	0.9 g/L
KH_2PO_4	0.5 g/L
$CaCl_2 \cdot 2H_2O$	0.1 g/L
$MgCl_2 \cdot 6H_2O$	0.2 g/L
$FeCl_2 \cdot 4H_2O$	0.1 g/L
NH_4Cl	0.5 g/L
$Na_2S.9H_2O$	0.5 g/L
$NaHCO_3$	3.5 g/L
1 g/L Resazurin Stock	1.0 mL/L
Vitamin Stock	1.0 mL/L
Trace Metal Stock	1.0 mL/L

Table 5.5. Composition of vitamin stock solution.

Vitamin Stock Solution	Concentration
Biotin	0.2 g/L
Folic Acid	0.2 g/L
Pyridoxine hydrochloride	1.0 g/L
Riboflavin	0.5 g/L
Thiamine	0.5 g/L
Nicotinic Acid	0.5 g/L
Pantothenic Acid	0.5 g/L
Vitamin B_{12}	0.01 g/L
p-Aminobenzoic Acid	0.5 g/L
Thioctic Acid	0.5 g/L

Table 5.6. Composition of sulfate-reducing culture feed.

Parameter	Concentration
pH	7.1 ± 0.2 [a]
Total COD, mg/L	2091 ± 28
Soluble COD, mg/L	300 ± 3
TSS, g/L	3.2 ± 0.05
VSS, g/L	1.3 ± 0.03
Ammonia, mg N/L	120
Phosphate Buffer (1:1,KH_2PO_4:K2HPO4; pH 7.2), mM	12
Sulfate, mg S/L	1479 ± 1.4
Anaerobic culture media, mL/L	100

[a]Mean ± std. dev., $\eta = 3$

after startup in batch assays. Serum bottles containing ASB sediment and anaerobic media were fed glucose, dextrin or lactate. Serum bottles fed glucose or dextrin generated significant gas production but serum bottles fed lactate did not.

5.2.1.3. Methanogenic Mixed Culture

A methanogenic mixed microbial culture was developed for use in experiments investigating biotransformation of phytosterols under methanogenic conditions. The culture was developed from an ASB sediment inoculum and fed pulp mill wastewater in order to simulate a methanogenic environment found in pulp mill ASB bottom sediments. The settled solids removed from the untreated pulp mill wastewater when making the aerobic culture feed were amended with nutrients (NH_4Cl to 120 mg N/L, phosphate buffer pH 7.2; 1:1, KH_2PO_4:K_2HPO_4 to 12 mM) and used as feed for the methanogenic culture. Characteristics of the methanogenic culture feed are given in Table 5.7. The presence of sulfate in the methanogenic culture feed is a result of the carryover of sulfate from the pulp mill wastewater. The culture was operated at a hydraulic retention time equal to the solids retention time of 21 days.

Table 5.7. Composition of methanogenic culture feed.

Parameter	**Concentration**
pH	7.1 ± 0.2 [a]
Total COD, mg/L	2091 ± 28
Soluble COD, mg/L	300 ± 3
TSS, g/L	3.2 ± 0.05
VSS, g/L	1.3 ± 0.03
Ammonia, mg N/L	120
Phosphate Buffer (1:1,KH_2PO_4:K2HPO4; pH 7.2), mM	12
Sulfate, mg S/L[b]	32.9 ± 1.4

[a]Mean ± std. dev., $\eta = 3$; [b] Carry over from the pulp mill wastewater

5.2.2. Bioenergetic Calculations

As a first step in determining the biotransformation potential of phytosterols in biological treatment systems, a group contribution method (Mavrovouniotis, 1991) was used to estimate the Gibb's free energy of each of the sterols used in this study. In this method, each fragment of the molecule is assigned a Gibb's free energy. The contributions of the fragments are summed, and corrections are included if certain structural features are present, to arrive at the estimated Gibb's free energy of the entire molecule (Equation 5.1). In equation 5.1, -23.6 is the origin $\Delta G^{0'}$ in kcal/mol for all molecules, a is the number of occurrences of group *i* and $\Delta G_i^{0'}$ is the standard Gibb's free energy of group *i*.

(Equation 5.1) $$\Delta G^{0\prime} = -23.6 + \sum_1^i a_i \, \Delta G_i^{0\prime} \text{ (kcal/mol)}$$

An oxidation half reaction for conversion of each sterol to CO_2 was formulated and combined with the reduction half reaction for the appropriate electron acceptor to calculate the Gibb's free energy of reaction under each redox condition.

5.2.3. Preliminary Assays With Stock Cultures

In order to evenly disperse the sterols within the biological system, it is necessary to introduce them in an already dissolved form. Ideally, water would be used as the solvent, because it would have no enhancing or inhibitory effects on the biotransformation potential of the sterols. However, the aqueous solubility of phytosterols is too low for water at neutral pH to make a suitable solvent. The aqueous solubility of some compounds increase when pH is either raised or lowered, thereby converting them to an ionic state, thus aiding their dissolution. The estimated pKa value of the sterol hydroxyl group is 15; thus, dissolution of sterols in a 50% NaOH solution

was attempted but was unsuccessful. Adding sterols to a biological system in a dry form or as an aqueous slurry was also attempted but these methods were also unsuitable. Sterols are hydrophobic and have a low density; thus, attempting to add sterols to the systems in an undissolved form left the sterols floating as crystals on the surface of the water until the sterol contacted the walls of the container. Then, sterols would travel up the meniscus, where they would settle on the walls of the reactor, above the water surface and out of contact with the system. It was then determined that an organic solvent was required to introduce the sterols into biological systems. Methanol was the first solvent tested, but cholesterol and phytosterols have limited solubility in methanol. Ethanol was then investigated for suitability. The solubility in ethanol of cholesterol and stigmasterol was tested using the stir flask method. The solubility in ethanol was determined to be 8 and 4 g/L for cholesterol and stigmasterol, respectively. Because ethanol would be added to microbial cultures, possible toxic and inhibitory effects of ethanol had to be first investigated.

5.2.3.1. Preliminary Assays with Aerobic Stock Culture

The acute toxicity of ethanol to the aerobic mixed microbial culture described in section 5.2.1.1 was investigated by performing oxygen uptake rate (OUR) experiments in the presence of glucose. The OUR of the culture was determined when 250 mg/L of glucose was present as sole carbon source. This value was used as the baseline OUR of the culture. The OUR of the culture was then determined when 1.65, 3.30 and 4.95 g COD/L of ethanol was added along with 250 mg/L glucose. The OUR under each of the conditions tested was evaluated and compared to the control (i.e., glucose only).

To perform the OUR experiments, the aerobic culture was aerated for one hour prior to the experiment in order for the dissolved oxygen (DO) concentration to be at saturation level (approximately 8.2 mg DO/L) at the beginning of the experiment. The aerobic culture, 25 mL, was transferred to a 30 mL Erlenmeyer flask, and a dissolved oxygen probe was inserted making sure that air did not remain inside the flask. The intersection of the probe and the flask was sealed with Parafilm to prevent air intrusion. The flask contents were stirred on a magnetic stirrer and the DO concentration was recorded over time.

As an additional preliminary experiment, the oxygen supply rate, K_{la}, was determined for the aerobic culture under aeration. The calculated rate is specific for the experimental setup and aeration rate used during the ethanol utilization and phytosterol biotransformation batch experiments. Tap water amended with $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ each to 60 mg/L, NH_4Cl to 60 mg N/L, phosphate buffer (pH 7.2; 1:1, KH_2PO_4:K_2HPO_4) to 30 mM and 1 mL of trace metals solution per liter (Table 5.2) was used as the liquid medium. Helium was bubbled through the medium for 30 minutes to strip DO. Aeration was initiated and DO was recorded over time until DO saturation was reached. The oxygen supply rate was determined as the initial slope of the DO vs. time curve after aeration was initiated.

A preliminary experiment was also carried out to investigate the utilization of ethanol by the aerobic stock culture under aerobic conditions in order to obtain DO and ethanol utilization data without interfering with the phytosterol biotransformation assay conducted later. Ethanol was added at an identical concentration to that used during the phytosterol batch biotransformation assay described in section 5.2.4.1, below. In this

experiment, 400 mL of aerobic culture was added to a 500 mL Erlenmeyer flask. Aeration of the culture was initiated and ethanol and ammonia were spiked to 0.5 mL ethanol/L (825 mg COD/L) and 60 mg N/L, respectively. DO, tCOD and VSS were monitored over time.

5.2.3.2. Ethanol Utilization by Sulfate-reducing Culture Assay

The ability of the sulfate-reducing mixed microbial culture to utilize ethanol as a carbon source was investigated using a preliminary batch assay. Sulfate-reducing culture (50 mL) was added to 160 mL helium-flushed serum bottles along with 48 mL of anaerobic media. Ethanol was spiked to 1 mL/L (1650 mg COD/L), ammonia to 120 mg N/L and sulfate to 1300 mg S/L. Gas pressure was monitored over the course of the experiment until gas production ceased and the experiment was terminated. Initial and final ethanol, acetate and sulfate were measured to assess whether sulfate-reduction and ethanol utilization occurred.

5.2.4. Batch Biotransformation Assays

5.2.4.1. Aerobic Batch Assays

Biotransformation of sterols by the aerobic mixed culture was investigated using a series of batch assays. Three separate experiments were performed. The first experiment was a biotransformation screening experiment which utilized cholesterol and stigmasterol as model sterols and consisted of a biotic degradation series and an abiotic control series. Once the biological transformation of the model sterols by the aerobic culture was confirmed, a second experiment was performed investigating the effect of N source on the rate and extent of cholesterol degradation. A final batch experiment was performed

to investigate the biodegradation of a phytosterol mixture. The experiment consisted of two biotic series: one containing the phytosterol mixture dissolved in ethanol, the other containing only ethanol. All experiments were carried out at 22°C and pH 7.

In the first experiment, 150 mL of aerobic culture was added to 250 mL Erlenmeyer flasks. Stock solutions were prepared of sodium azide in DI water (5 g/L), ammonium chloride in DI water (30 g N/L), cholesterol in ethanol (8 g/L) and stigmasterol in ethanol (4 g/L). Half of the flasks were set aside to serve as abiotic controls and were spiked with 200 mg/L sodium azide to inhibit any biological activity. After the sodium azide had been allowed to contact the abiotic control flasks for 1 hour, ammonium chloride was added to 150 mg N/L and cholesterol and stigmasterol were each added to 4 mg/L in all flasks. The flasks were agitated on an Orbital shaker at 190 rpm for 10 days. Samples were taken periodically during the experiment and extracted to determine remaining sterol concentrations.

The second experiment was carried out in 500 mL Erlenmeyer flasks aerated by compressed, pre-humidified air. Aerobic culture (400 mL) was added to each flask and either NH_4Cl or $NaNO_3$ was added to 60 mg N/L. Cholesterol stock solution was added to each flask to a final concentration of 8 mg cholesterol/L. Aeration was provided by compressed, pre-humidified air, and the flasks were stirred with a magnetic stir plate and Teflon-coated stir bar. The flask containing nitrate was amended with nitrate a second time to maintain a significant nitrate concentration in the system. The flask containing ammonia was amended with ammonia daily due to its rapid removal by ammonia-oxidizing bacteria. A corresponding increase in nitrate was observed within this system as a result of nitrification. The ammonia containing flasks were adjusted to pH 7

periodically due to the drop in pH that occurred as a result of ammonia oxidation (i.e., nitrification). The flask contents were sampled periodically and sterol, nitrate and ammonia concentrations were determined.

The third experiment was carried out in 500 mL Erlenmeyer flasks aerated with compressed, pre-humidified air. Stirring was provided by a magnetic stir plate. Aerobic culture (400 mL) was added to each flask and amended with ammonium chloride to 60 mg N/L. A stock solution of the phytosterol mixture in ethanol was prepared and contained campesterol, stigmasterol and β-sitosterol at 1.25, 1.26 and 2.06 g/L, respectively. Each flask was amended with either 0.5 mL of the phytosterol stock solution or 0.5 mL of ethanol, and the flasks were aerated for 6 days. The flasks were sampled, and sterols, ammonia, nitrate and soluble COD concentrations were measured periodically during the experiment.

5.2.4.2. Nitrate-reducing Batch Assay

A batch assay was carried out which utilized the aerobic stock culture under nitrate-reducing conditions. This assay was carried out at 22°C and pH 7 in a 1.5 L reactor equipped with ports for sampling headspace and liquid contents. The aerobic culture (1 L) was settled for 2 hours, and 0.67 L of the supernatant was removed and replaced with aerobic culture feed. The culture was then transferred to the reactor. The reactor was sealed with a rubber stopper, and the headspace was flushed with helium for 20 minutes. Sodium nitrate was added to 30 mg N/L. The culture was mixed on a magnetic stir plate overnight and nitrate was measured to confirm that nitrate reduction had occurred. The phytosterol mixture stock solution was added to 0.5 mL/L, and 120 mg N/L of sodium nitrate was added. Initial samples were taken for phytosterol, sCOD

and nitrate measurements. An additional 0.5 mL/L of the phytosterol mixture stock solution was added on days 7 and 14. Additional nitrate was added to 120 mg N/L on day 7 and to 180 mg N/L on days 14 and 21. Headspace pressure was measured and liquid samples were taken for phytosterols, sCOD and nitrate measurement once per week. The experiment was terminated after 5 weeks.

5.2.4.3. Sulfate-reducing and Methanogenic Batch Assays

Assays utilizing the sulfate-reducing and methanogenic mixed cultures were carried out in 160 mL glass serum bottles sealed with rubber stoppers and aluminum crimps. Both assays were carried out at 22°C and pH 7. The two assays were conducted according to the same experimental protocol, described below. Replicate serum bottles were prepared in pairs and opened periodically, 5 pairs to test biological degradation and 3 pairs to test abiotic degradation for each culture. The serum bottles were sealed and flushed with helium for 20 minutes. The culture (60 mL) and culture feed (30 mL) were added to each serum bottle. The feed used for these assays was identical to the feed of the corresponding stock culture, as described in sections 5.2.1.2 and 5.2.1.3, above. Serum bottles used for abiotic controls were amended with sodium azide to 200 mg/L to inhibit biological activity. The sodium azide was allowed to contact the culture for 2 days before addition of phytosterols. Phytosterol mixture stock solution (0.05 mL) and 10 mL of DI water were added to each serum bottle. The serum bottles were mixed daily by hand during the experiment, while the liquid contents of the bottle were not allowed to contact the rubber stopper during mixing. Serum bottles were opened after 0, 3, 5, 7 and 10 weeks for the biotic series and after 0, 5 and 10 weeks for the abiotic series. Headspace pressure was monitored throughout the incubation period. Before opening the

serum bottles, headspace composition and pressure were measured. After opening the bottles, liquid contents were sampled for phytosterol measurement. Remaining ammonia was measured after 10 weeks for both culture series. Remaining sulfate after 10 weeks was measured in the sulfate-reducing culture series.

5.3. Results and Discussion

5.3.1. Stock Culture Performance

5.3.1.1. Aerobic Stock Culture Performance

Two successive feeding cycles of the aerobic stock culture, performed 10 months after its initial development, were characterized to demonstrate steady-state operation and results are shown in Figure 5.1. Table 5.8 gives performance characteristics for the aerobic stock culture during a one week feeding cycle. Initial and final average pH was 7.3 and 6.7. The pH drop was a result of ammonia oxidation. The culture was able to degrade 191 mg sCOD/L or 64% of influent sCOD during a feeding cycle. Most (77%) of the added ammonia was oxidized to nitrate and the rest was used for the production of biomass. Ammonia was not detected in the culture effluent (i.e., settled mixed liquor supernatant).

5.3.1.2. Sulfate-reducing Stock Culture Performance

Typical steady-state culture characteristics are given in Table 5.9. Gas production of the sulfate-reducing culture during one feeding cycle is shown in Figure 5.2. The total gas produced (186 mL at 22^{o}C) was composed of 70, 25 and 5 % CO_2, H_2S and CH_4, respectively. The culture degraded 38 % of tCOD and 29% of sulfate present in the feed.

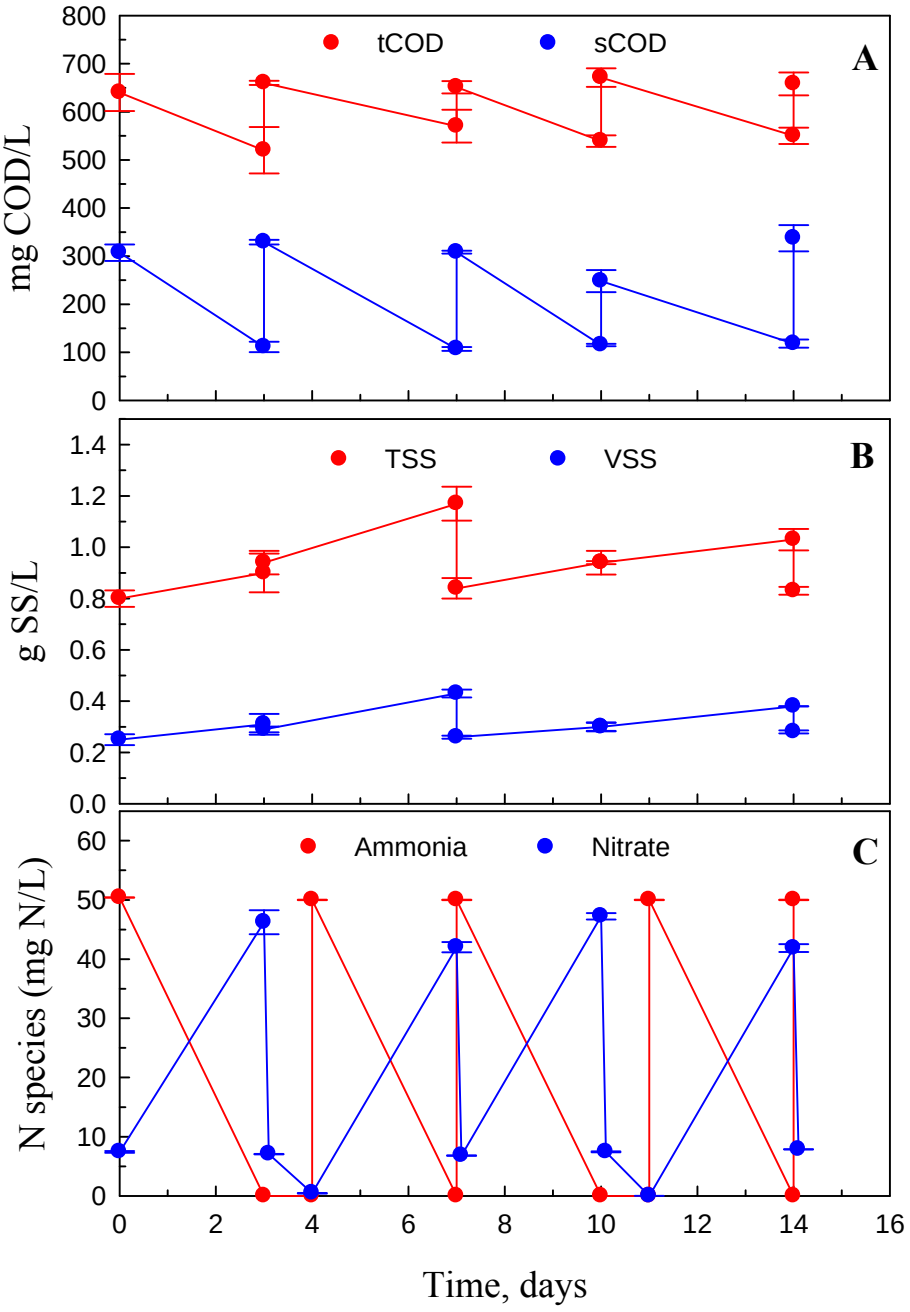


Figure 5.1. Characterization of 2 week-long feeding cycles for the aerobic mixed culture (Error bars represent one standard deviation of the mean).

Table 5.8. Characteristics of an aerobic culture feeding cycle (Effluent and mixed liquor values given for day 7 of feeding cycle).

Parameter	Influent	Effluent[a]	Mixed Liquor
pH	7.3 ± 0.2 [a]	6.7 ± 0.2	6.7 ± 0.2
Total COD, mg/L	351 ± 5	170 ± 8	584 ± 30
Soluble COD, mg/L	300 ± 3	109 ± 10	109 ± 10
TSS, g/L	0.1 ± 0.05	0.2 ± 0.01	1.1 ± 0.02
VSS, g/L	0.1 ± 0.02	0.1 ± 0.01	0.4 ± 0.03
Ammonia, mg N/L	60 ± 2	ND	ND
Nitrate, mg N/L	ND	46 ± 2	46 ± 2

[a] Settled mixed liquor supernatant

Table 5.9. Characteristics of a sulfate-reducing culture feeding cycle.

Steady-state Parameter	Value[a]
CH_4 Production, mL @ 22°C	7.8 ± 0.002
CO_2 Production, mL @ 22°C	131.4 ± 12.9
H_2S Production, mL @ 22°C	47.2 ± 3.5
TS, g/L	15.0 ± 0.5
VS, g/L	4.0 ± 0.6
Total COD removal, mg COD/L	801 ± 36
Sulfate utilization, mg S/L	437 ± 127

[a]Mean ± std. dev., $\eta = 3$

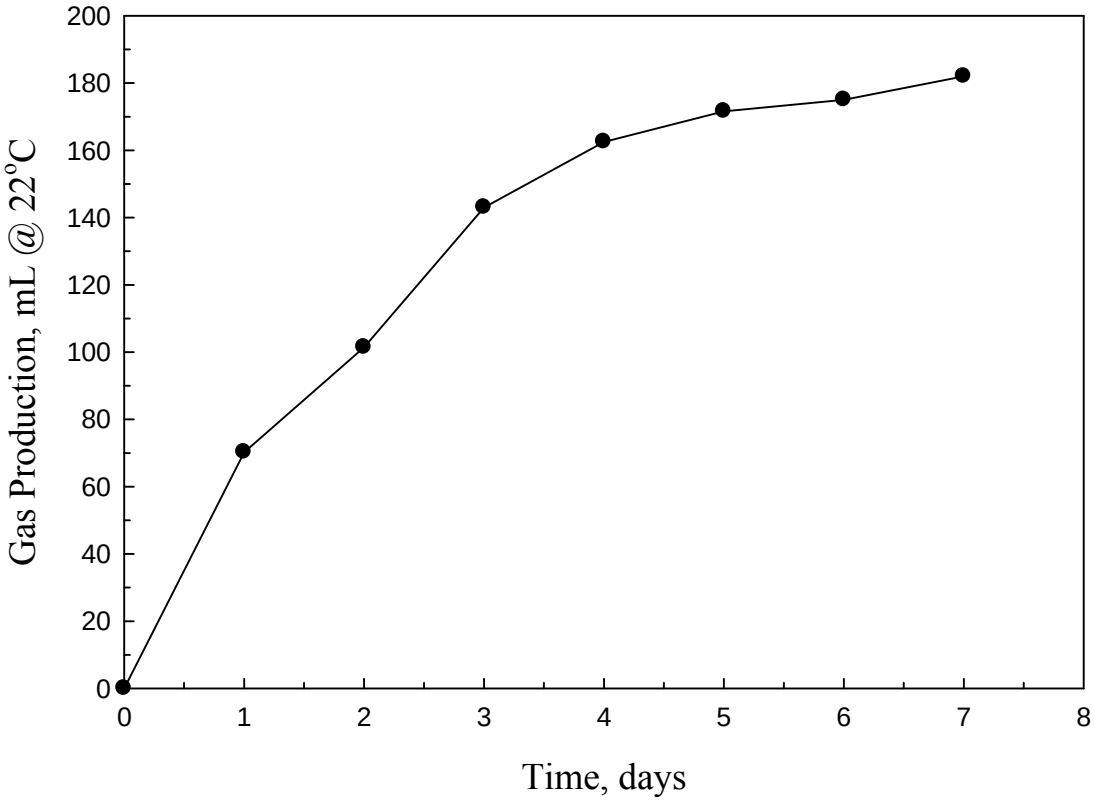


Figure 5.2. Total gas production during one feeding cycle of the sulfate-reducing stock culture.

5.3.1.3. Methanogenic Stock Culture Performance

Typical steady-state culture characteristics of the stock methanogenic culture are given in Table 5.10. Gas production of the methanogenic culture during one feeding cycle is shown in Figure 5.3. The total gas produced (165 mL at 22°C) was composed of 47 % CH_4 and 53 % CO_2. The culture degraded 35 % of tCOD present in the feed.

5.3.2. Aerobic Stock Culture Phytosterol Removal

The concentrations of individual phytosterols in the aerobic stock culture feed, liquid phase and mixed liquor are shown in Table 5.11. Calculated mixed liquor and

Table 5.10. Characteristics of a Methanogenic Culture Feeding Cycle.

Steady-state Parameter	**Value**[a]
CH_4 Production, mL@22°C	77.8 ± 7.6
CO_2 Production, mL@22°C	87.7 ± 20.1
TS, g/L	12.1 ± 0.08
VS, g/L	4.3 ± 0.31
Total COD removal, mg COD/L	731 ± 54.6

[a]Mean ± std. dev., $\eta = 3$

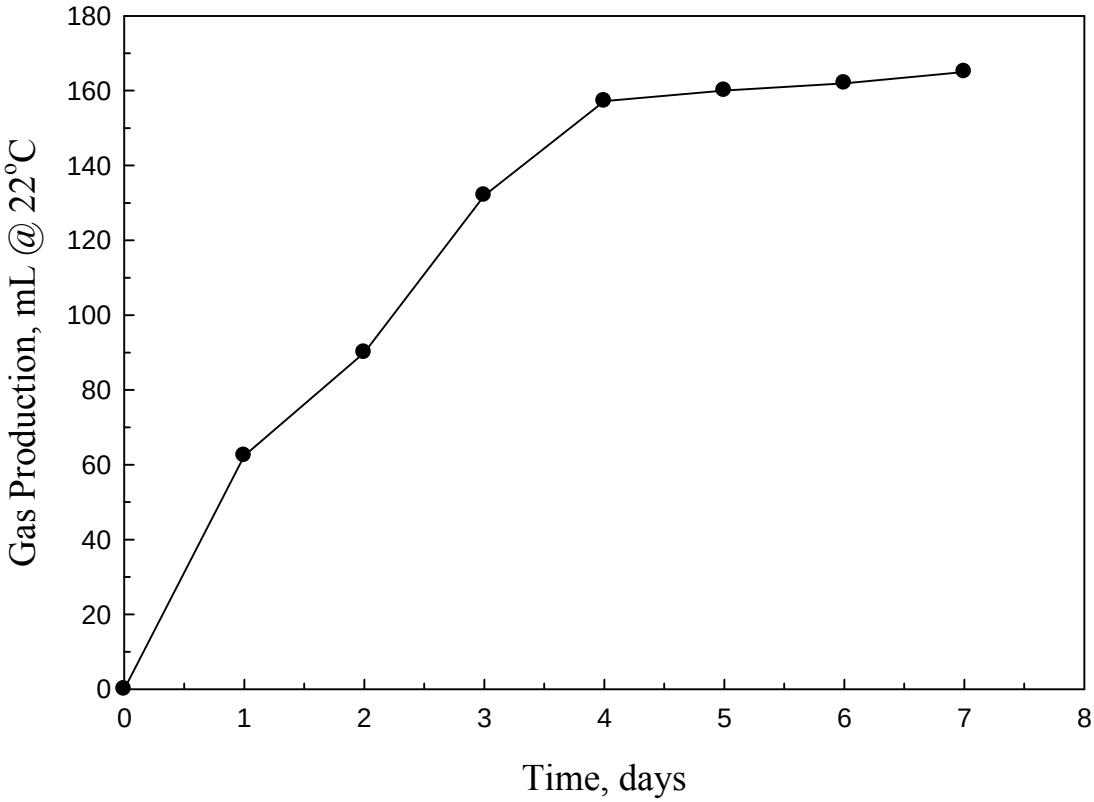


Figure 5.3. Total gas production during one feeding cycle of the methanogenic stock culture.

Table 5.11. Phytosterol concentrations for aerobic mixed culture.

Phytosterol	Feed	Liquid Phase[b]	Mixed Liquor
Campesterol, µg/L	3.5 ± 0.3[a]	1.7 ± 0.2	10.7 ± 6.1
Stigmasterol, µg/L	2.2 ± 0.1	2.5 ± 0.1	14.0 ± 6.1
β-sitosterol, µg/L	32.0 ± 1.5	3.2 ± 0.3	46.6 ± 19.6

[a]Mean ± std. dev., $\eta = 2$; [b]Supernatant after mixed liquor settling

liquid phase concentrations of phytosterols, normalized to the feed concentration, are plotted versus time in Figure 5.4. Phytosterols were assumed to partition according to the Freundlich partitioning constants for stigmasterol which were calculated in Chapter 4, section 4.3.2.1 (K_f = 0.334 (µg/mg VSS)(L/µg)*n; n = 0.463). During the aerobic culture operation, 33 % of the total mixed liquor was wasted every 7 days, and 75 % of the total culture liquid phase (settled culture supernatant) was wasted twice every 7 days. The up and down trend of the phytosterol concentrations are associated with the cyclical wasting and feeding of the culture. At steady-state, the concentration of phytosterols in the mixed liquor of the aerobic culture is estimated to be 4.2 times the feed concentration. The predicted steady-state mixed liquor concentrations of the phytosterols, assuming no biodegradation, falls within the range measured in the mixed liquor for campesterol and stigmasterol indicating that phytosterol biodegradation was not significant. The predicted mixed liquor concentration of β-sitosterol, however, is approximately twice the highest measured concentration of β-sitosterol. It is difficult to assign the discrepancy to biotransformation because of the large standard deviation in the β-sitosterol measurement. It is most likely that phytosterols are not biodegraded by the aerobic stock culture at these very low concentrations. As expected, the phytosterol mixed liquor concentrations are much greater than the concentrations in the liquid phase, indicating

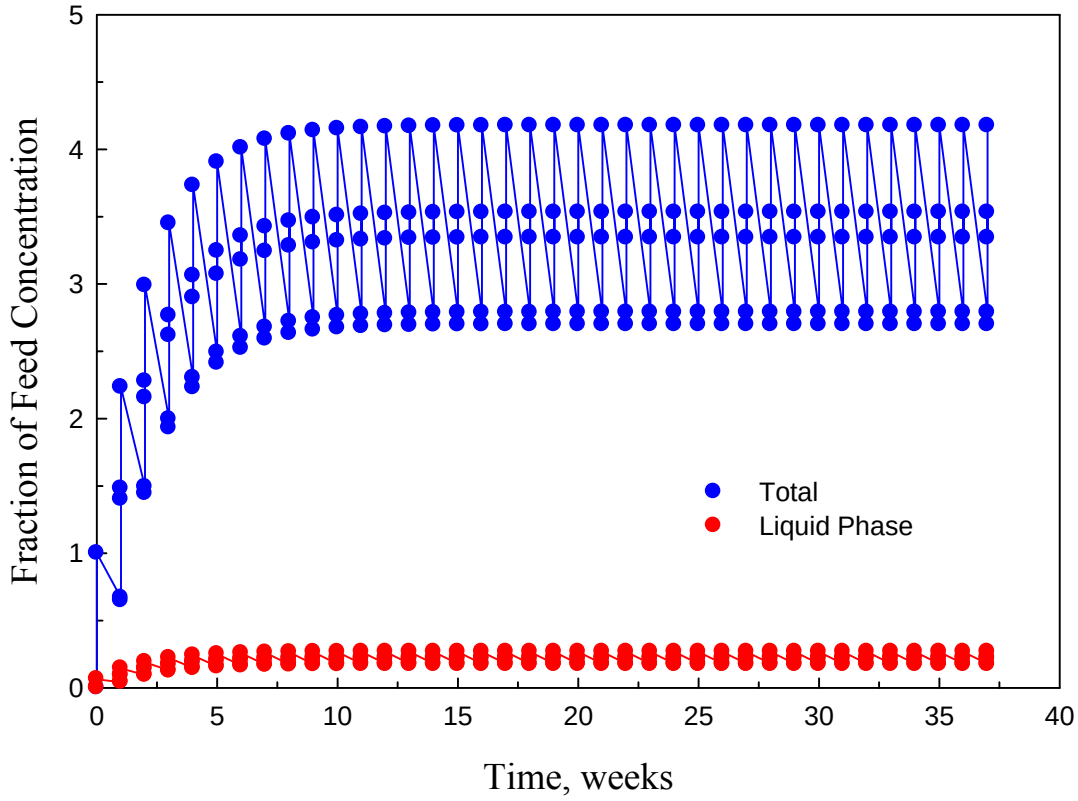


Figure 5.4. Calculated liquid phase and total mixed liquor concentrations of phytosterols, normalized to the feed concentration.

that most of the phytosterols are adsorbed to solids. At steady-state, the concentration of phytosterols in the liquid phase is estimated to be 6 % of the total mixed liquor concentration. The measured phytosterol concentrations in the liquid phase were 16, 18 and 7 % of the mixed liquor concentrations of campesterol, stigmasterol and β-sitosterol, respectively. The liquid phase concentrations of campesterol and stigmasterol are significantly higher than predicted, but the concentration of β-sitosterol is very close to the predicted value.

5.3.3. Energetics of Sterol Biodegradation

The estimated Gibb's free energy for cholesterol, campesterol, stigmasterol and β-sitosterol are 54.81, 87.87, 173.64 and 94.98 kJ/mol, respectively. Details for the Gibb's free energy calculations are provided in Appendix A. The estimated standard Gibb's free energy for the sterol reduction half-reactions are given in Table 5.12. The Gibb's free energy for sterol reduction half-reactions is between 25.99 and 26.59 kJ/eeq. The similarity in the Gibb's free energy for the sterol half-reactions arises because, even though structural differences between the sterols lead to different values for Gibb's free energy, the difference is negligible when divided by the large number of electron equivalents (eeq) in the entire molecule, which is between 151 and 164. The Gibb's free energy for the oxidation reactions for each sterol under aerobic, nitrate-reducing, sulfate-reducing and methanogenic conditions were calculated according to Equation 5.2., below. Results are shown in Table 5.13 for β-sitosterol. The Gibb's free energy values for the oxidation of the other sterols under the redox conditions tested are summarized in Appendix B. From a purely energetic standpoint, biodegradation is theoretically favorable for the sterols under each of the redox conditions of interest.

(Equation 5.2) $$\Delta G_r' = \Delta G_{ea}^{0\prime} - \Delta G_{ed}^{0\prime} \text{ (kJ/eeq)}$$

5.3.4. Preliminary Assays with Stock Cultures

5.3.4.1 Preliminary Assays with Aerobic Culture

The results of the aerobic culture OUR experiments are shown in Figure 5.5. The presence of 1.65 g COD/L of ethanol enhanced the OUR rate above what was observed with glucose and no ethanol present; however, the presence of 3.30 and 4.95 g COD/L of

Table 5.12. Standard Gibb's free energy of sterol reduction half-reactions at pH 7 and 298K.

Half-reactions	$\Delta G^{0'}$ kJ/eeq
β-sitosterol: $\frac{29}{164}CO_2 + H^+ + e^- \leftrightarrow \frac{1}{164}C_{29}H_{50}O + \frac{57}{164}H_2O$	26.27
Campesterol: $\frac{28}{158}CO_2 + H^+ + e^- \leftrightarrow \frac{1}{158}C_{28}H_{48}O + \frac{55}{158}H_2O$	26.27
Stigmasterol: $\frac{29}{162}CO_2 + H^+ + e^- \leftrightarrow \frac{1}{162}C_{29}H_{48}O + \frac{57}{162}H_2O$	26.59
Cholesterol: $\frac{27}{151}CO_2 + H^+ + e^- \leftrightarrow \frac{1}{151}C_{29}H_{50}O + \frac{53}{151}H_2O$	25.99

Table 5.13. Standard Gibb's free energy of β-sitosterol oxidation reactions under different redox conditions at pH 7 and 298K.

Redox Condition	$\Delta G_r'$ kJ/eeq
Aerobic $C_{29}H_{50}O + 41O_2 \leftrightarrow 29CO_2 + 25H_2O$	-104.99
Nitrate-reducing $C_{29}H_{50}O + 32.8NO_3^- + 32.8H^+ \leftrightarrow 29CO_2 + 41.4H_2O + 16.4N_2$	-98.47
Sulfate-reducing $C_{29}H_{50}O + 20.5SO_4^{2-} + 30.75H^+ \leftrightarrow$ $29CO_2 + 25H_2O + 10.25H_2S + 10.25HS^-$	-5.42
Methanogenic $C_{29}H_{50}O + 16H_2O \leftrightarrow 8.5CO_2 + 20.5CH_4$	-2.74

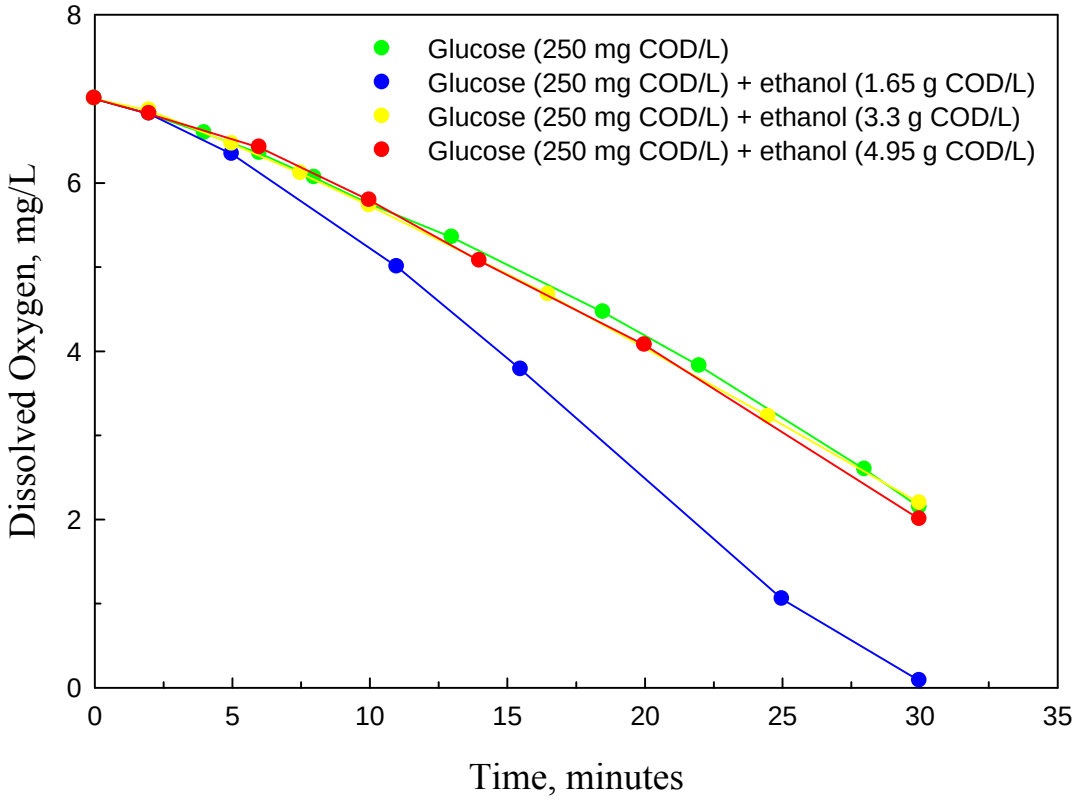


Figure 5.5. Effect of ethanol on the OUR of the aerobic culture amended with 250 mg glucose/L and ethanol at three different levels.

ethanol had no enhancing effect on OUR. Thus, the presence of ethanol had no observable negative effect on OUR by the aerobic culture.

The results of the experiment used to determine the K_{la} of the aerobic batch degradation experimental setup are shown in Figure 5.6. As the medium DO was being stripped with helium, the DO decreased before reaching a steady state of approximately 0.8 mg DO/L. When aeration was initiated at the 30th minute, the DO level rapidly rose before slowing as DO approached saturation. The slope of the initial rise was used to calculate the K_{la} of the experimental setup, 1007 mg DO/L·d.

DO, tCOD and VSS data from the ethanol utilization experiment are shown in Figure 5.7. Direct measurement of ethanol was not possible at the time this experiment occurred. Total COD was determined to be a suitable proxy and was used to determine when ethanol utilization was complete. The decrease in tCOD degradation rate and the recovery of DO to saturation level indicates that ethanol utilization was completed within 25 hours or earlier. Most of the microbial growth occurred during the first 10 hours. The DO profile indicates that oxygen levels are reduced to near zero after 10 hours as a result of the biological activity. The calculated K_{la} for the experimental setup was 1007 mg DO/L·d, and the concentration of ethanol used during the experiment, 0.5 mL/L, was equivalent to 825 mg COD/L. Therefore, sufficient oxygen should be supplied to the system within the first 20 hours to completely degrade the added ethanol without depriving the culture of DO. The data support this estimate.

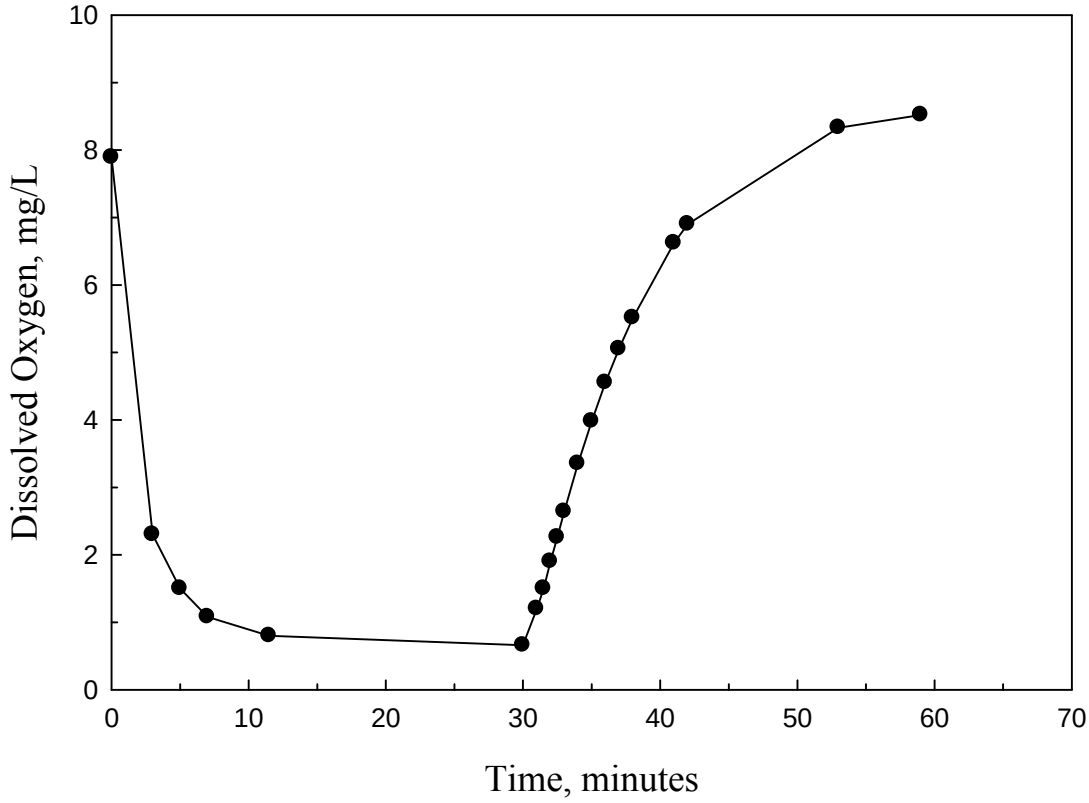


Figure 5.6. Dissolved oxygen profile observed during the experimental determination of the K_{la} of the aerobic experimental setup (DO stripping followed by aeration).

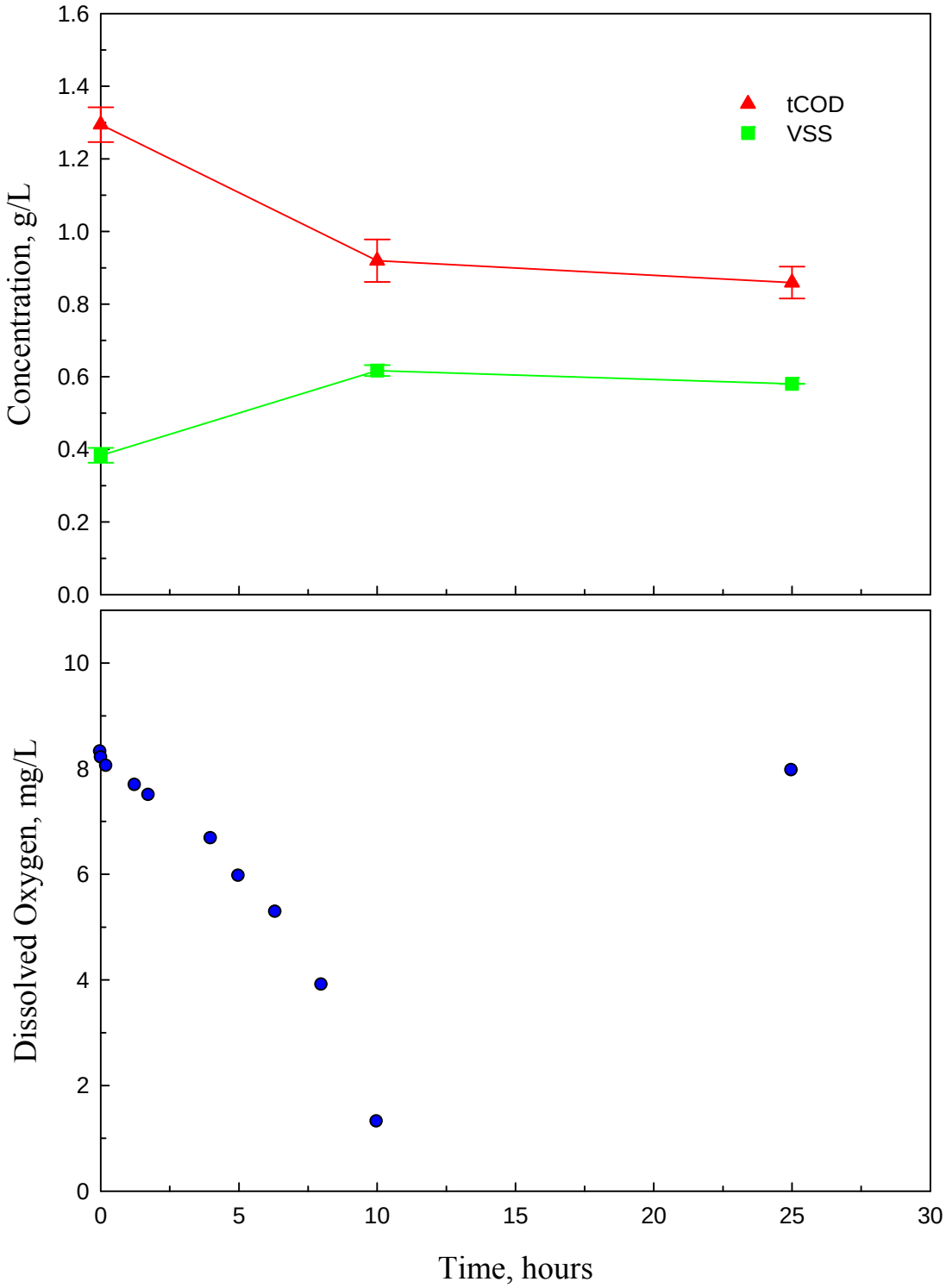


Figure 5.7. DO, tCOD and VSS during the ethanol utilization by the aerobic stock culture under aerobic conditions (Error bars represent one standard deviation of the mean).

5.3.4.2. Preliminary Assay with Sulfate-reducing Culture

Initial and final sulfate, acetate and ethanol data for the test (i.e., biotic) and control (i.e., abiotic) series are shown in Table 5.14. Total gas production for both series is shown in Figure 5.8. The stoichiometric equation for ethanol utilization during sulfidogenesis using ethanol as the electron donor is given in Equation 5.3, below.

(Equation 5.3) $C_2H_5OH + 0.5SO_4^{2-} \rightarrow CH_3COO^- + 0.5HS^- + 0.5H^+ + H_2O$

According to Equation 5.3, utilization of 1 mole of ethanol requires 0.5 moles of sulfate and results in the production of 1 mole of acetate. In this assay, sulfate was observed to decrease in a 0.5:1 mole ratio to ethanol utilization. A corresponding 1:1 mole increase in acetate was also observed. The control series exhibited a negligible amount of gas production attributed to atmospheric pressure variation; however, ethanol decrease, acetate increase or sulfate decrease was not observed in the abiotic series. These results clearly indicate that the sulfate-reducing culture is capable of utilizing ethanol as an electron donor during sulfidogenesis.

Table 5.14. Ethanol, acetate and sulfate data for ethanol utilization during the sulfidogenesis experiment.

Analyte	**Test (Biotic)**		**Control (Abiotic)**	
	Initial	**Final**	**Initial**	**Final**
Ethanol, mM	31.5 ± 1.1	0.0 ± 0.0	30.3 ± 0.7	29.8 ± 0.2
Acetate, mM	0.13 ± 0.02	32.3 ± 0.05	0.57 ± 0.01	0.32 ± 0.11
Sulfate, mM	39.5 ± 3.8	22.5 ± 2.1	42.7 ± 7.1	42.4 ± 2.9

[a]Mean ± std. dev., n = 2

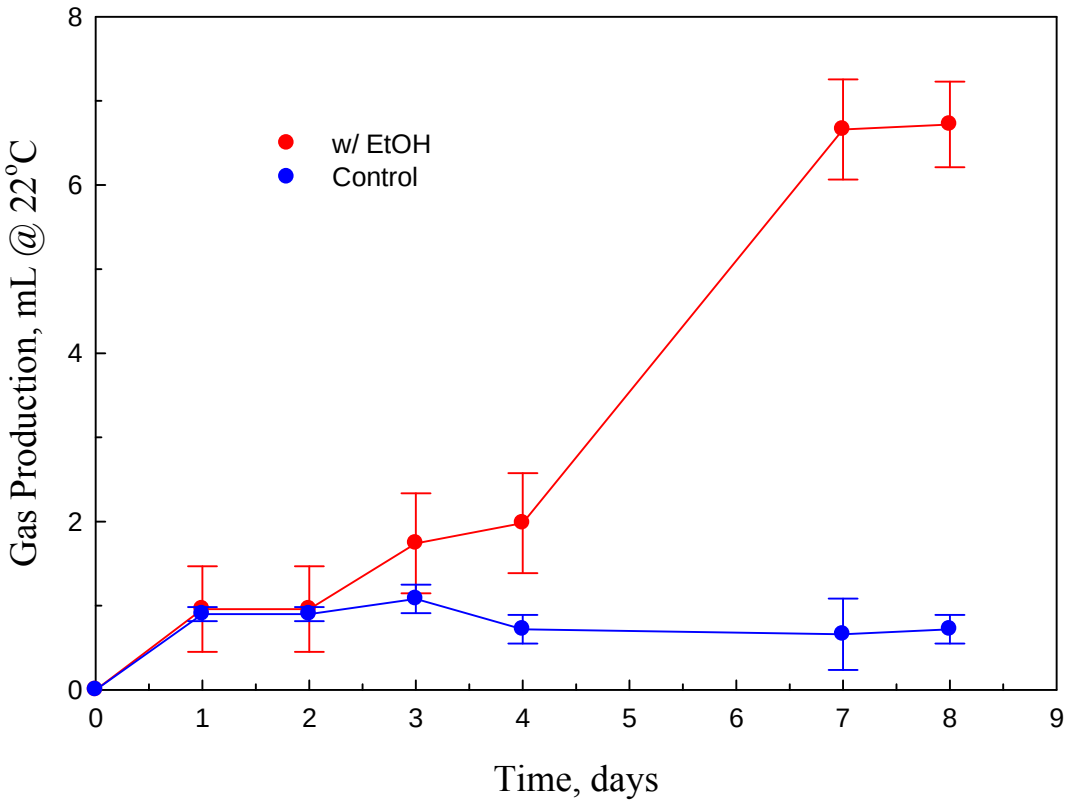


Figure 5.8. Gas production with ethanol utilization during the sulfidogenesis experiment (Error bars represent one standard deviation of the mean).

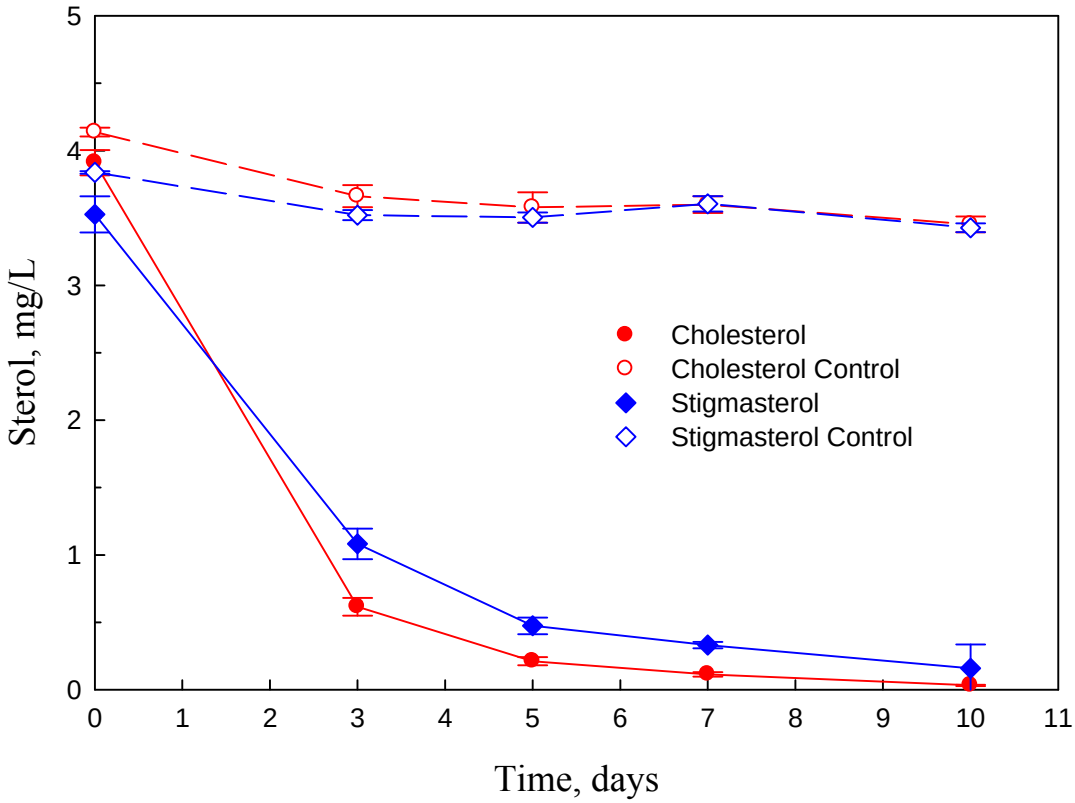


Figure 5.9. Sterol concentration during the aerobic degradation screening experiment (Error bars represent one standard deviation of the mean).

5.3.5. Biotransformation Potential Under Aerobic Conditions

5.3.5.1. Assay Results

Sterol concentrations during the aerobic sterol degradation screening experiment are shown in Figure 5.9. The biotic series was sampled daily and the pH was adjusted to 7 using a 1 N sodium bicarbonate solution. Neither cholesterol nor stigmasterol degraded in the abiotic control series. However, in the biotic series, both cholesterol and stigmasterol degraded from approximately 4 mg/L to the low μg/L range within 10 days. Degradation of both sterols was rapid during the first 3 days of incubation before slowing. These data demonstrate that cholesterol and stigmasterol are both biotransformed at about the same rate and extent by the aerobic stock culture under aerobic conditions.

After biotransformation of sterols by the aerobic stock culture under aerobic conditions was confirmed, the effect of nitrogen source on the cholesterol degradation rate was investigated in a batch assay. Ammonia is the more energetically favorable nitrogen source for protein synthesis due to its lower redox state, -3 compared to nitrate at +5. A lower redox state means that fewer electron equivalents are required for the synthesis of biomass, nitrogen redox state -3, from ammonia.

Ammonia, nitrate and cholesterol data are presented in Figure 5.10. The additional nitrate seen in both series at the beginning of the experiment was present in the aerobic stock culture, which contained 46 mg N/L nitrate as a result of nitrification during normal operation. Additional ammonia was added daily to the ammonia-amended series to maintain significant levels of ammonia in the system. Additional nitrate had to be added once to the nitrate-amended series after the first day. Cholesterol degraded at

the same rate and to the same extent in both series, which indicates the aerobic culture degraded cholesterol at the same rate and extent regardless of which inorganic nitrogen source was present.

The aerobic biotransformation of a mixture of phytosterols by the aerobic stock culture was investigated in a batch assay. Ammonia, nitrate, pH, sCOD and phytosterol data are shown in Figure 5.11. As a result of nitrification, the pH decreased from 7.29 to 6.85 after 1 day in both the ethanol only and ethanol and phytosterol-amended series. The pH was adjusted to pH 7 with a 1 N sodium bicarbonate solution, and no further adjustment was required. No differences were observed in the ammonia utilization or nitrate production rates between the two series, indicating that the presence of phytosterols did not affect the nitrification rate. Concentrations of all three phytosterols decreased rapidly during the first 10 hours of the assay. The rate of degradation slowed afterward and degradation practically stopped after 48 hours. The assay was continued for an additional 4 days, after which the mean phytosterol concentrations were 9.7, 8.9 and 18.9 μg/L for campesterol, stigmasterol and β-sitosterol, respectively.

Sterol degradation is most likely induced by the presence of another easily degradable carbon source. This would mean that biotransformation of phytosterols is the result of either co-metabolism, where the organism cannot directly use phytosterols for energy, or of secondary metabolism, i.e., phytosterol degradation is achieved when another, degradable substrate is present (i.e., primary substrate), which supports primary metabolism. The degradation of the phytosterols and removal of sCOD was observed to follow a similar trend during the aerobic degradation assays. Each aerobic experiment used a different combination of sterols and different starting concentrations; thus, the

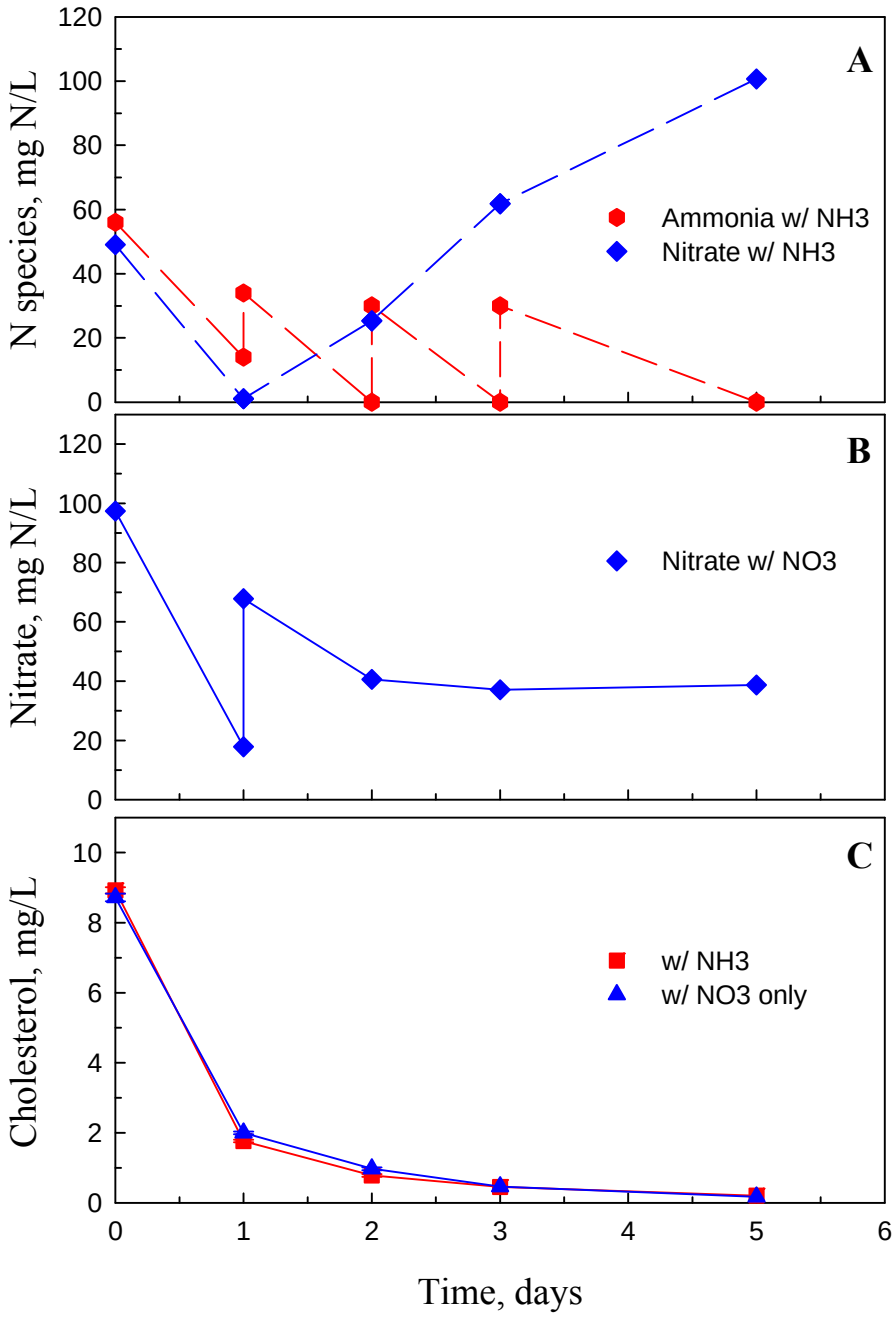


Figure 5.10. Data from aerobic stock culture assay assessing the effect of inorganic N source. (A) N species from ammonia-amended assay; (B) N species from nitrate-amended assay; (C) Cholesterol from both assays.

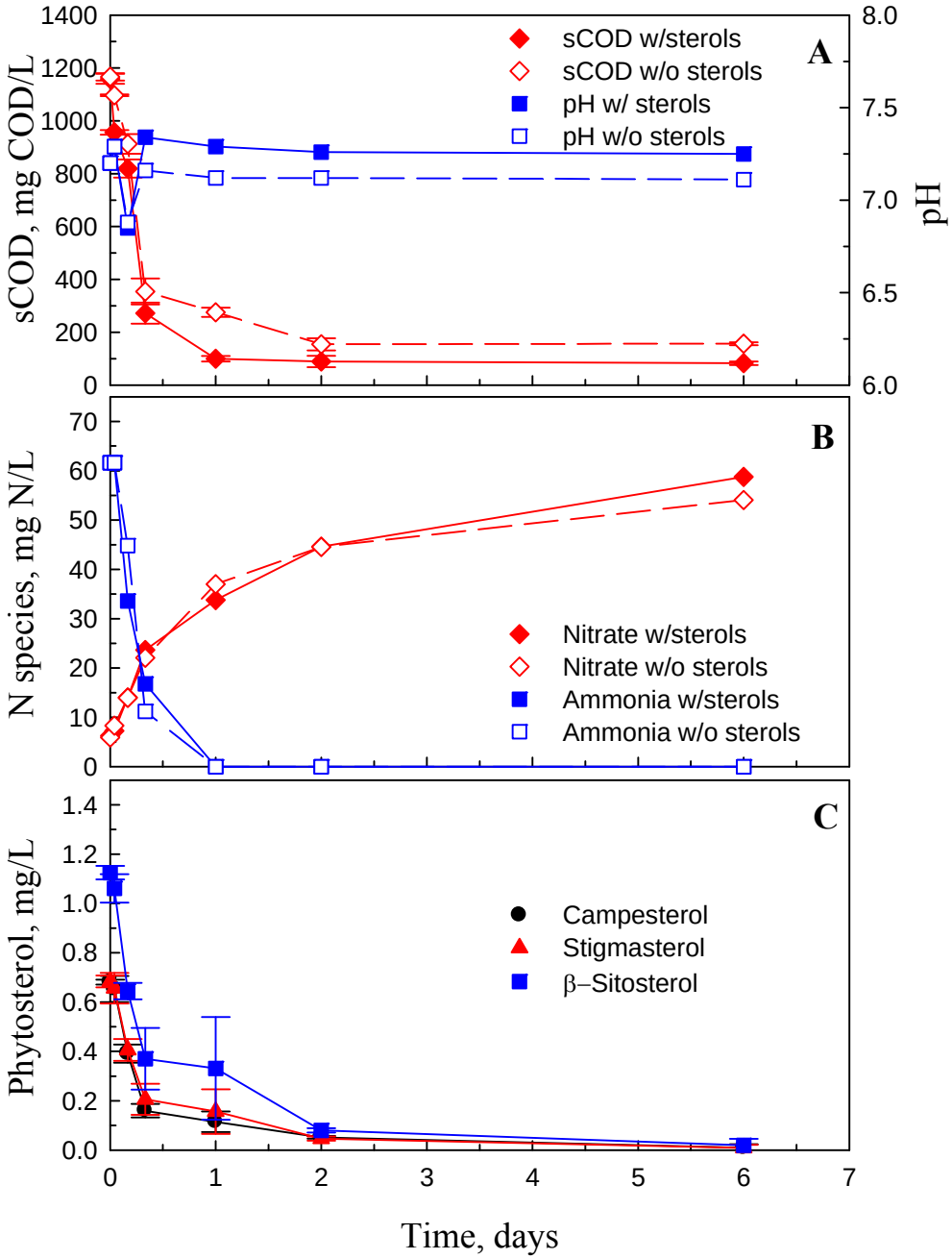


Figure 5.11. Data from the aerobic phytosterol biotransformation assay. (A) Soluble COD and pH; (B) Ammonia and nitrate; (C) Phytosterol from the phytosterol-amended series (Error bars represent one standard deviation of the mean).

evidence is indirect. Also, in the experiment evaluating the preference for inorganic nitrogen source, sCOD was not measured directly; however, by observing the rate of nitrate utilization in the nitrate-amended series, the trend of sCOD utilization during the assay can be acquired. Utilization of sCOD would be accompanied by a corresponding decrease in nitrate; thus, the trend in sCOD degradation should mirror the trend in nitrate removal.

In both of these experiments, sCOD was degraded rapidly at the beginning of the experiment followed by a lower rate. Eventually, sCOD was no longer degraded. Rapid phytosterol degradation was observed to accompany rapid sCOD degradation. The sterol degradation rate also decreased when the sCOD degradation slowed. Once sCOD degradation had ceased, sterols continued to degrade, but at a very low rate. These observations are indicative of either co-metabolic or secondary degradation of phytosterols, both requiring the presence of a degradable carbon and energy source. Given the necessity of using ethanol as solvent to dispense sterols, delineation between phytosterol co-metabolic or secondary degradation was not feasible in this study. However, from the practical point of view of pulp mill wastewater treatment, given the fact that phytosterols exist at very low (µg/L range) concentrations in the presence of relatively high (g/L range) concentrations of a mixture of degradable organic compounds, it is encouraging that phytosterols are degradable under aerobic conditions typically encountered in engineered treatment systems.

5.3.4.2. Kinetics

The rate of biotransformation of phytosterols may be affected by such factors as the initial concentration of phytosterols and the concentration of the primary substrate.

Toxicity or inhibition as a result of phytosterol concentration is not expected to be an issue. The biotransformation rate of phytosterols can be described by a Michaelis-Menten-type equation:

(Equation 5.3.) $$-\frac{dC}{dt} = \frac{kXC}{K_S+C}\frac{P}{K_P+P}$$

where C is the phytosterol concentration (mg/L), k is the maximum phytosterol biotransformation rate per unit biomass (mg (mg of VSS)$^{-1}$ d^{-1}), X is the phytosterol biotransforming biomass concentration (mg of VSS/L), K_s is the half-velocity coefficient for phytosterol biotransformation (mg/L), P is the primary substrate(s) concentration (mg/L), and K_P is the half velocity coefficient for primary substrate use (mg/L). The concentration of primary substrate (i.e., ethanol) was kept in excess as compared to the phytosterol concentration. Biomass increase during the batch assay was consistently less than 10%. Under the imposed experimental conditions and because the specific phytosterol degrading biomass concentration could not be measured, the biotransformation rate of phytosterols was described by the following equation:

(Equation 5.4) $$-\frac{dC}{dt} = \frac{k'C}{K_S+C}$$

where k' = kX ≈ constant, is the maximum phytosterol biotransformation rate (mg L^{-1} d^{-1}) for a constant biomass concentration and other imposed experimental conditions as discussed above. Integration of Equation 5.4 yields:

(Equation 5.5) $$C = C_0 - K_S\ln(C/C_0) - k't$$

In this form, the dependent variable C is a function of C/C_0; thus, the equation cannot be solved explicitly for C. Equation 5.5 was re-written so that t is the dependent variable in order to obtain an equation with a unique solution:

(Equation 5.6) $$t = [C_0 - C - K_S \ln(C/C_0)]/k'$$

Using non-linear regression (SigmaPlot) Equation 5.6 was used to find the best fit of model parameters K_s and k' to the experimental data of C given the measured C_0 values. A Michaelis-Menten half-lifetime equation was derived by evaluating Equation 5.4 for the condition of $S = S_0$:

(Equation 5.7) $$t_{1/2} = \frac{0.5C_0 - K_S \ln(0.5)}{k'}$$

The Michaelis-Menten half-lifetime expression (Equation 5.7) is dependent on the initial contaminant concentration, C_0. Therefore, comparisons of the phytosterol biotranformation rates of phytosterols using Equation 5.5 are only meaningful when applied at the same initial phytosterol concentration.

Maximum phytosterol biotransformation rates (k'), half-velocity coefficients (K_s) and half-lifetimes ($t_{1/2}$) for each phytosterol tested are given in Table 5.15. Experimental data and model predictions based on the Michaelis-Menten model are shown in Figure 5.12. Campesterol and stigmasterol were added to similar initial concentrations and were biotransformed at similar rates. The rate of biotransformation of β-sitosterol was similar to those observed for campesterol and stigmasterol even though β-sitosterol was added at 65 % higher initial concentration than the other two sterols. Predicted values of K_s had a high degree of uncertainty which is typical for low K_s values. In contrast, there is higher confidence for predicted values of k'.

5.3.6. Biotransformation Potential Under Nitrate-reducing Conditions

Initial and final pH during the assay was 7.2 and 7.4, respectively. Average gas production was 430 ± 73 mL@22^{o}C/week. Nitrate, sCOD and phytosterol data are

Table 5.15. Michaelis-Menten kinetic coefficients for aerobic phytosterol degradation.

Phytosterol	k', mg L^{-1} d^{-1}	K_s, mg L^{-1}	r^2	$t_{1/2}$, h
Campesterol	5 ± 1.5[a]	1.8 ± 1.0[a]	0.890	7.7
Stigmasterol	5 ± 1.5	1.8 ± 1.0	0.917	7.7
β-sitosterol	5 ± 1.5	2.5 ± 1.0	0.863	11.0

[a]Mean ± std. dev., $\eta = 3$

shown in Figure 5.13. After each addition of phytosterol stock solution, an increase in soluble COD was observed due to the COD contribution of ethanol. The culture degraded most of the added COD within one week; however, a recalcitrant COD fraction remained and accumulated. When sCOD as ethanol addition ceased (day 21), a reduction in the recalcitrant portion of COD then occurred.

Nitrate was removed within one week of each nitrate addition when the phytosterol stock solution was also added. Phytosterol concentrations were unchanged after the first week. A reduction in phytosterol concentration was observed after the second week and an even greater reduction was observed after the third week. This pattern appeared to follow enrichment of phytosterol degraders after each addition of phytosterol leading to a greater degree of degradation after each subsequent phytosterol addition. If the phytosterol data was evidence of biodegradation and microbial enrichment, then a further decrease in phytosterol concentration would be expected during the fourth week, which was initiated with addition of nitrate and no further addition of phytosterols and thus no ethanol. However, this was not the case. If, however, the reasoning put forth in the previous section, that sterol degradation occurs by co-metabolism or secondary metabolism, holds true, then the decrease during the fourth week would not be expected to be significant. Nitrate was removed between day 21 and

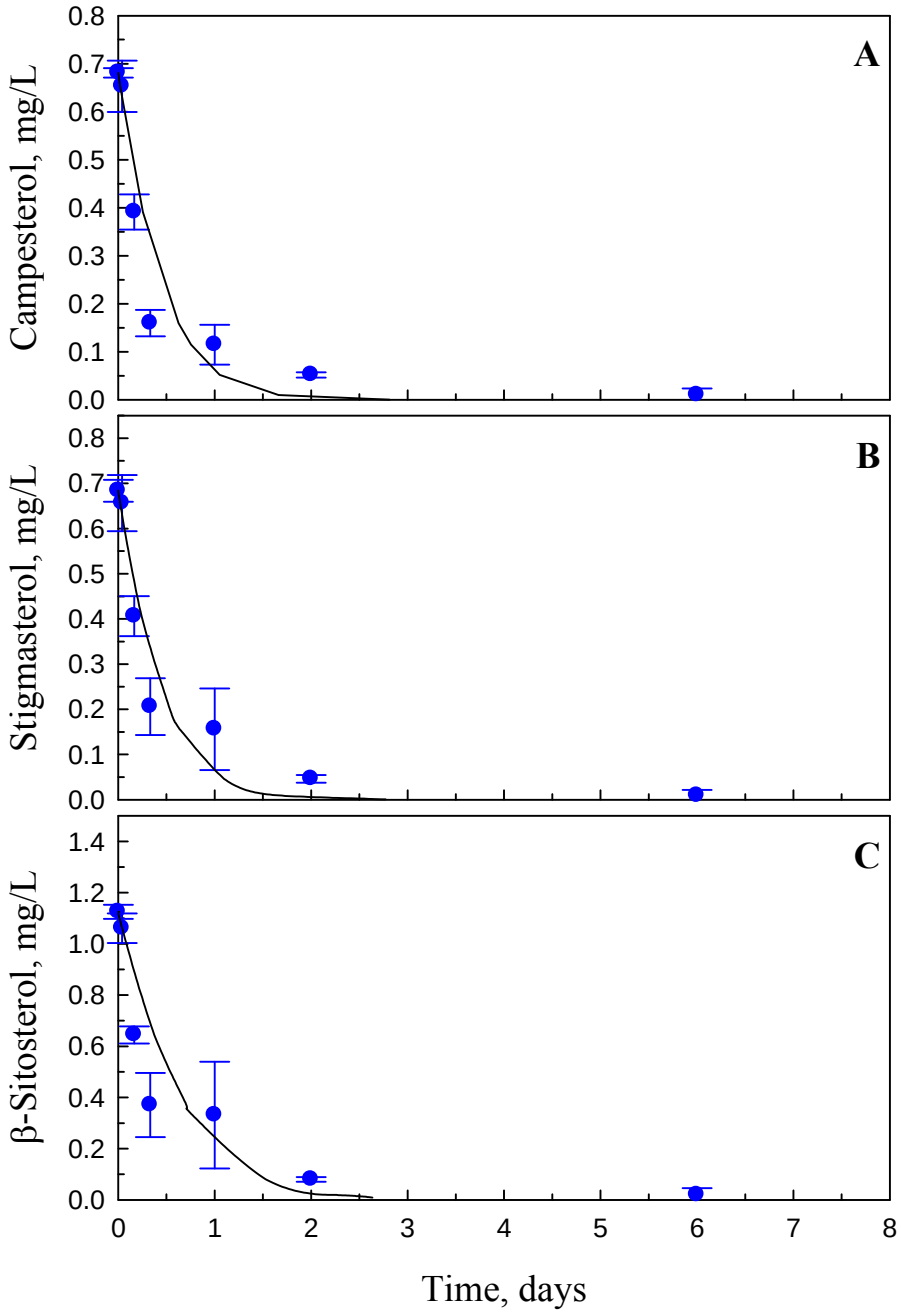


Figure 5.12. Time course of (A) campesterol, (B) stigmasterol and (C) β-sitosterol biotransformation during a batch assay. Lines are model predictions based on the Michaelis-Menten model (Error bars represent one standard deviation of the mean).

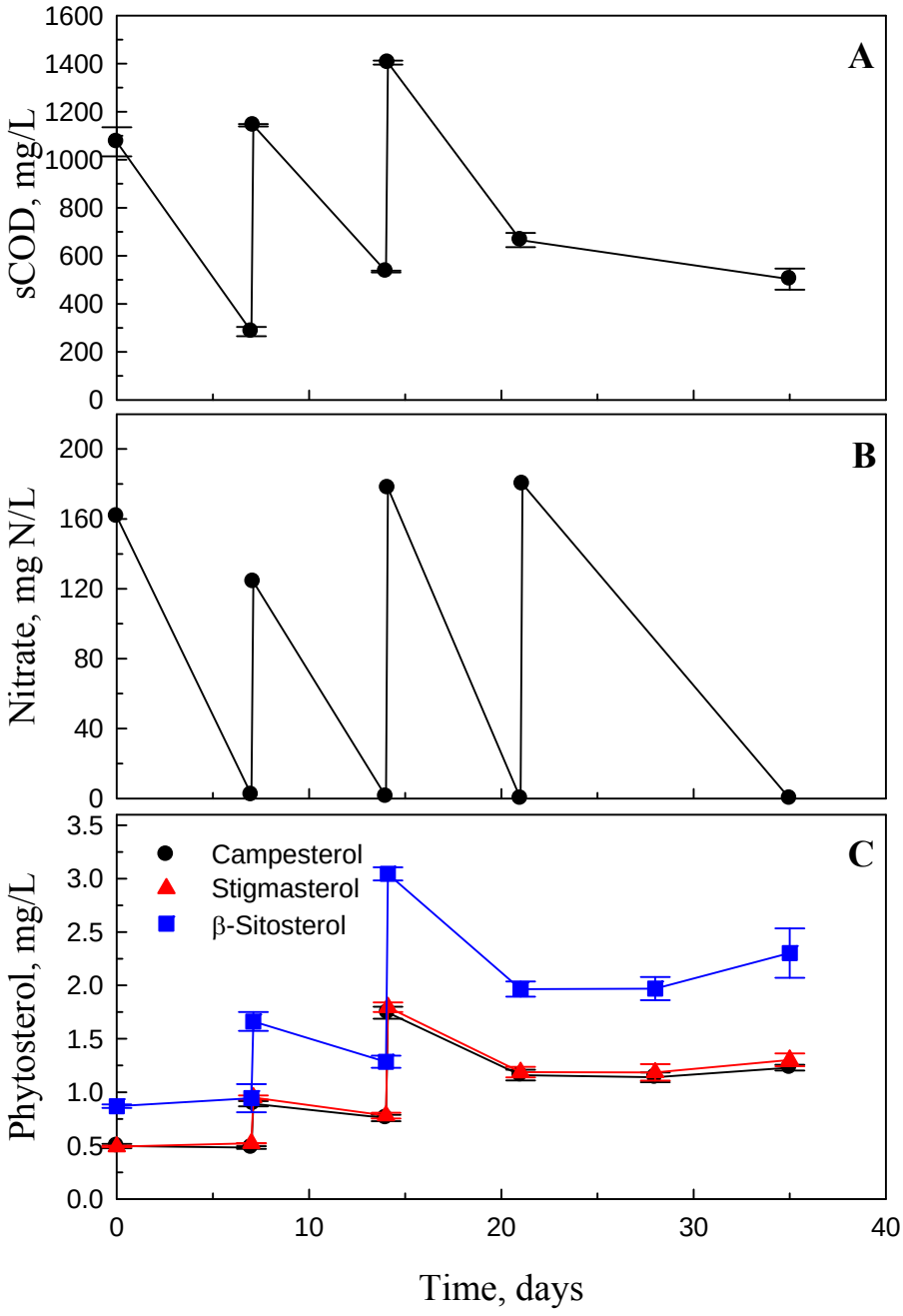


Figure 5.13. Data from the batch phytosterol biotransformation assay under nitrate-reducing conditions. (A) sCOD; (B) Nitrate; (C) Phytosterols (Error bars represent one standard deviation of the mean).

day 35 even though additional sCOD was not added on day 21. The COD:N ratio for nitrate utilization during the first two weeks was 4.9; however in week 3 the ratio dropped to 4.2. If a ratio of COD:N of 4.9 is assumed for utilization of nitrate by the stock culture, then the decrease in nitrate of 180 mg N/L observed to occur between days 21 and 35 should have been accompanied by a decrease in tCOD of 882 mg COD/L; however, the observed drop in sCOD was only 160 mg COD/L. The additional COD required for the total utilization of nitrate must have come from degradation (i.e., decay) of biomass, which was not measured. The increase in phytosterol concentration observed during the fifth week cannot be explained in terms of degradation because no additional sterols were added. The error bars, however, do overlap with the error bars from day 28 which indicates that statistically, there was no change in phytosterol concentration between day 21 and the end of the assay. Biodegradation of cholesterol under nitrate-reducing conditions has been reported in the literature although not by cultures derived from a pulp mill biological treatment system (Harder and Probian, 1997; Chiang et al., 2008).

5.3.7. Biotransformation Potential Under Sulfate-reducing Conditions

Initial and final pH during the assay was 7.3 and 7.3, respectively. Residual ammonia in the assay was 61.2 ± 1.1 mg N/L after 10 weeks and residual sulfate was 282.6 ± 1.6 mg S/L. Data collected during the assay are shown in Figure 5.14. Gas was produced during the first 2 weeks of the assay after which no gas production occurred. Gas composition was 64% carbon dioxide, 31% hydrogen sulfide and 5% methane at day 21 and remained constant after gas production ceased. Phytosterol concentrations increased initially in the test series, by 64 % in the case of β-sitosterol, before falling

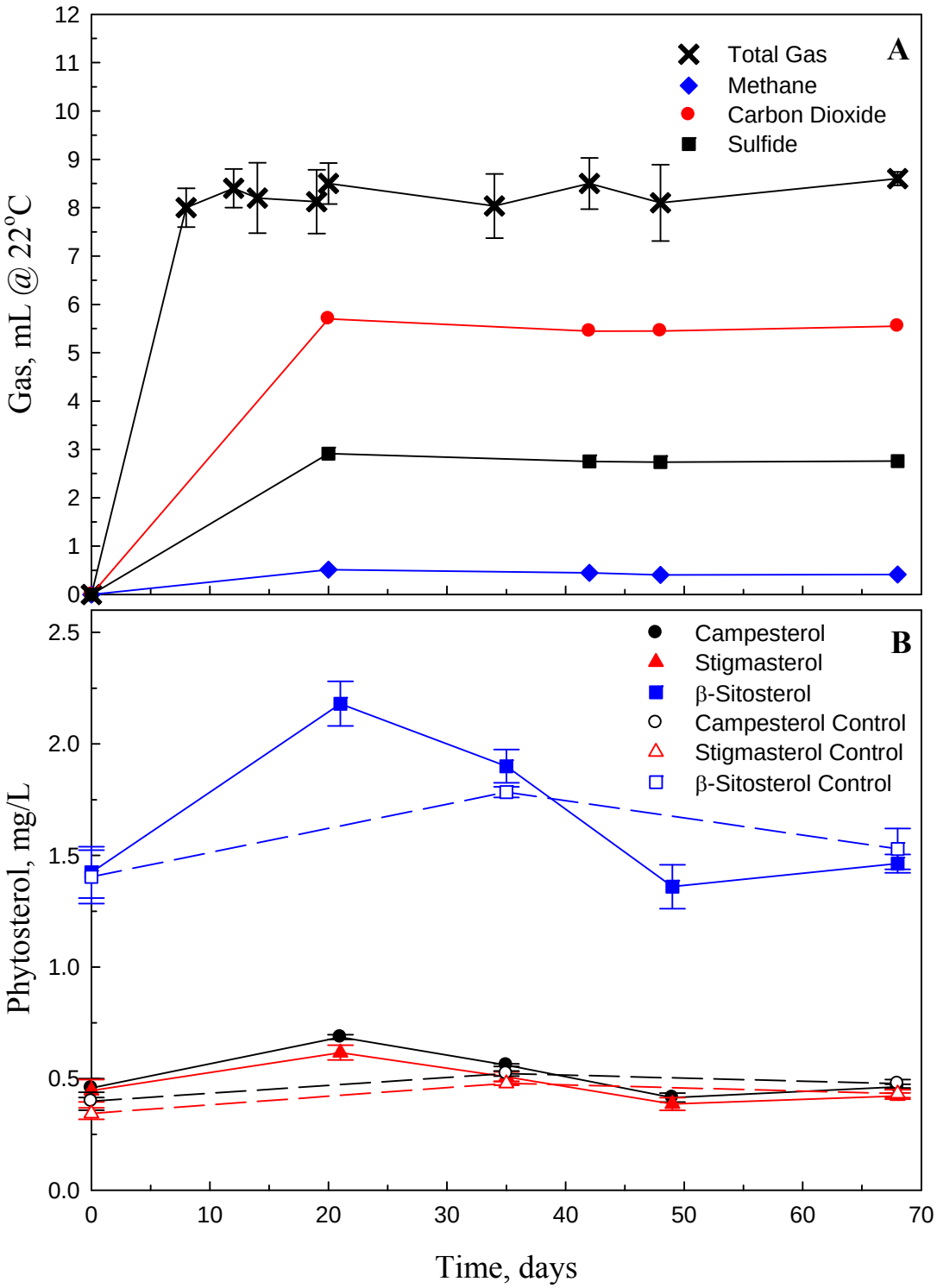


Figure 5.14. Data from phytosterol batch degradation assay under sulfate-reducing conditions (Error bars represent one standard deviation of the mean).

back to the initially measured levels. The initial increase in measured phytosterols could be due to improved recovery of the phytosterols as a result of wastewater solids degradation, which would have been most significant during the initial 2 weeks of the assay. If this were the case, however, a subsequent decrease in the phytosterol concentrations would not be expected. Phytosterol concentrations in the abiotic control series also increased initially before returning to the original values. This could not be due to solids degradation as biological activity was inhibited in the abiotic series. The up and down trend in measured phytosterol concentrations is proportional for all sterol measurements in both the biotic and abiotic series, which indicates the presence of a systematic error of unknown origin. This trend was also observed in the methanogenic assay (section 5.3.8, below). Comparison of starting and final phytosterols concentrations in the test series indicates that biotransformation of phytosterols during the assay did not occur. A previous experiment utilizing a culture derived from marine sediments also failed to show that cholesterol was biodegraded under sulfate-reducing conditions (Taylor et al., 1981).

5.3.8. Biotransformation Potential Under Methanogenic Conditions

A decrease in pH from pH 7.2 to pH 6.7 was observed during the incubation period. Residual ammonia was 67.2 ± 0.3 mg N/L after 10 weeks. Data collected during the assay are shown in Figure 5.15. Gas was produced most rapidly during the first week of the assay followed by a period of slower production until week 7, after which gas production ceased. The gas was composed of 60 % methane and 40 % carbon dioxide. Similarly to what was observed in the sulfate-reducing assay, phytosterol concentrations were observed to increase initially; however, the increase was not as pronounced, 40 %

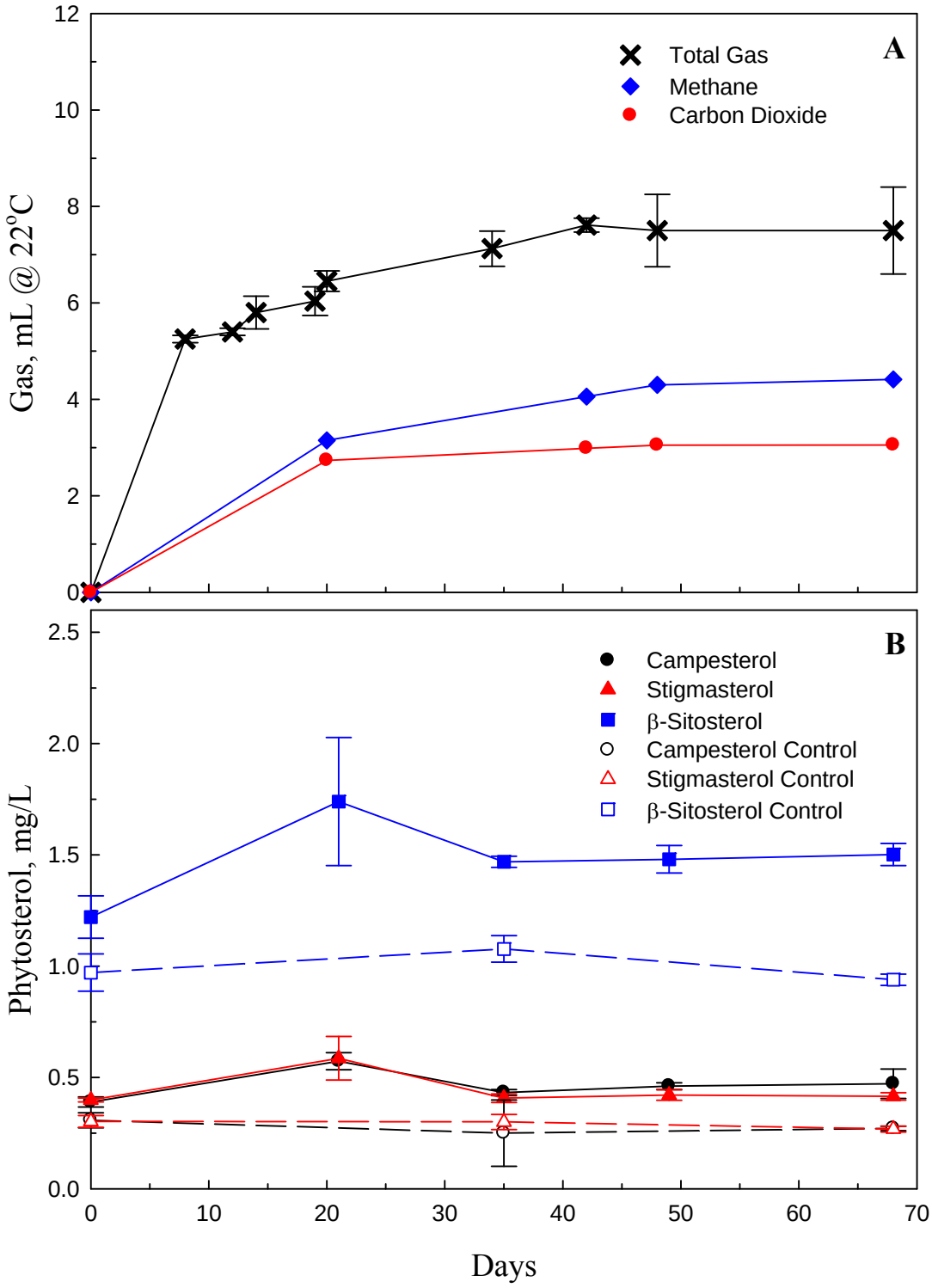


Figure 5.15. Data from phytosterol batch degradation assay under methanogenic conditions (Error bars represent one standard deviation of the mean).

increase in β-sitosterol compared to 64 % in the sulfate-reducing assay. The overall trend in phytosterol concentrations did not fluctuate as much as during the sulfate reduction assay but also point to a systematic error. After the initial increase occurred, a slight downward trend was observed, before the phytosterol concentrations leveled off at slightly more than their original value. No decrease in phytosterol concentrations were observed in the biotic and abiotic series indicating that phytosterols were not degraded by biological or abiotic means under methanogenic conditions. A hydrogenation reaction of cholesterol to cholestanol by fermentative bacteria was observed in anaerobic systems (Gaskell et al., 1975). Degradation of cholesterol or phytosterols by methanogens has not been reported in the literature.

5.4. Summary

Mixed microbial cultures were developed from inoculum taken from a pulp mill ASB and fed untreated pulp mill wastewater. Each culture was operated under either aerobic/denitrifying, sulfate-reducing or methanogenic conditions and steady-state characterizations were made. Using a group contribution method the standard Gibb's free energy of selected sterols was calculated and used to perform theoretical energetic calculations. The oxidation of phytosterols under aerobic, nitrate-reducing, sulfate-reducing and methanogenic conditions was determined to be energetically favorable. Batch assays were conducted at 22°C and pH 7 to investigate the biotransformation potential of phytosterols under these redox conditions. Phytosterol biotransformation was observed only under aerobic conditions. The biotransformation rate under aerobic conditions was not dependent on the source of nitrogen provided but was dependent on the availability of an easily degradable carbon source, indicating that aerobic

Phytosterol biotransformation did not occur under sulfate-reducing or methanogenic conditions. The results of the assay performed under nitrate-reducing conditions showed a decrease in phytosterols concentrations, but biotransformation could not be conclusively shown. The phytosterols initially appeared to be degraded following a microbial enrichment pattern, but ceased degrading when ethanol was not present. The findings of the batch assays are consistent with what has been reported in the literature for the biotransformation of cholesterol.

CHAPTER 6

CONTINUOUS-FLOW SYSTEM

6.1. Introduction

Aerated stabilization basins (ASBs) cannot be considered homogeneous systems. Surface aerators provide a moderate degree of mixing of the water column; however, wastewater solids and biomass settle out of the mixed liquor, forming a bottom sediment. The main terminal microbial processes occurring in these sediments are sulfate-reduction and methanogenesis (Mahmood, 2008). Phytosterols enter ASBs with the wastewater with a significant fraction adsorbed to wastewater solids, mainly cellulose. Once they enter the ASB, they may desorb from the wastewater solids and become dispersed in the water column either in freely dissolved form or adsorbed to dissolved organics. They may also adsorb to suspended biomass or remain adsorbed to wastewater solids and, if they are not degraded, settle out of suspension. The biotransformation potential of phytosterols by the various microbial consortia that they may be exposed to within an ASB has been investigated in batch assays, as discussed in the previous chapter (Chapter 5). However, the effect of the interplay between the various physical and microbial processes cannot be inferred from simple batch assays.

The objective of the research reported in this chapter was to assess the phase distribution and biotransformation potential of phytosterols in biological systems incorporating multiple environments using a continuous-flow biological treatment system.

6.2. Materials and Methods

A continuous-flow system was developed from ASB mixed liquor. A schematic of the reactor is shown in Figure 6.1. The reactor had a liquid volume of 7 L and was initially filled with ASB mixed liquor collected at a pulp mill plant. Characteristics of the mixed liquor are given in Chapter 5, Table 5.1. Untreated pulp mill wastewater was used as feed for the system. The wastewater was stored at 4°C until it was combined with the other feed components. After the feed was mixed it was stored at 3°C until being fed to the system. Nitrogen and phosphorus were added to the feed as NH_4Cl (30 mg N/L) and KH_2PO_4 (10 mg P/L), respectively. Feed characteristics are given in Table 6.1. The hydraulic retention time of the system was 21 days. The feed was pumped by a positive displacement FYI Lab Pump at 35 mL/min, timed to operate for 1.5 minutes every 4 hours, which delivered a total flow rate of 315 mL/d. The wastewater solids from the feed were allowed to accumulate in the reactor and formed a bottom sediment layer. Aeration was provided by compressed, pre-humidified air forced through glass pipettes which were submerged 4 cm below the water surface. Algal growth was prevented by wrapping the reactor in aluminum foil.

Untreated pulp mill wastewater, amended with 30 mg N/L and 10 mg P/L was fed to the system continuously for 14 months. Solids were allowed to accumulate in the reactor forming a sediment layer composed of wastewater solids and biomass. The sediment reached a steady-state volume of 1L with a porosity of approximately 0.3. After several months of operation, the sediment developed a black coloration, which is attributed to the presence of metal sulfides as a result of sulfate-reduction occurring in the sediment. Total sulfide in the sediment was measured and sulfate degradation was

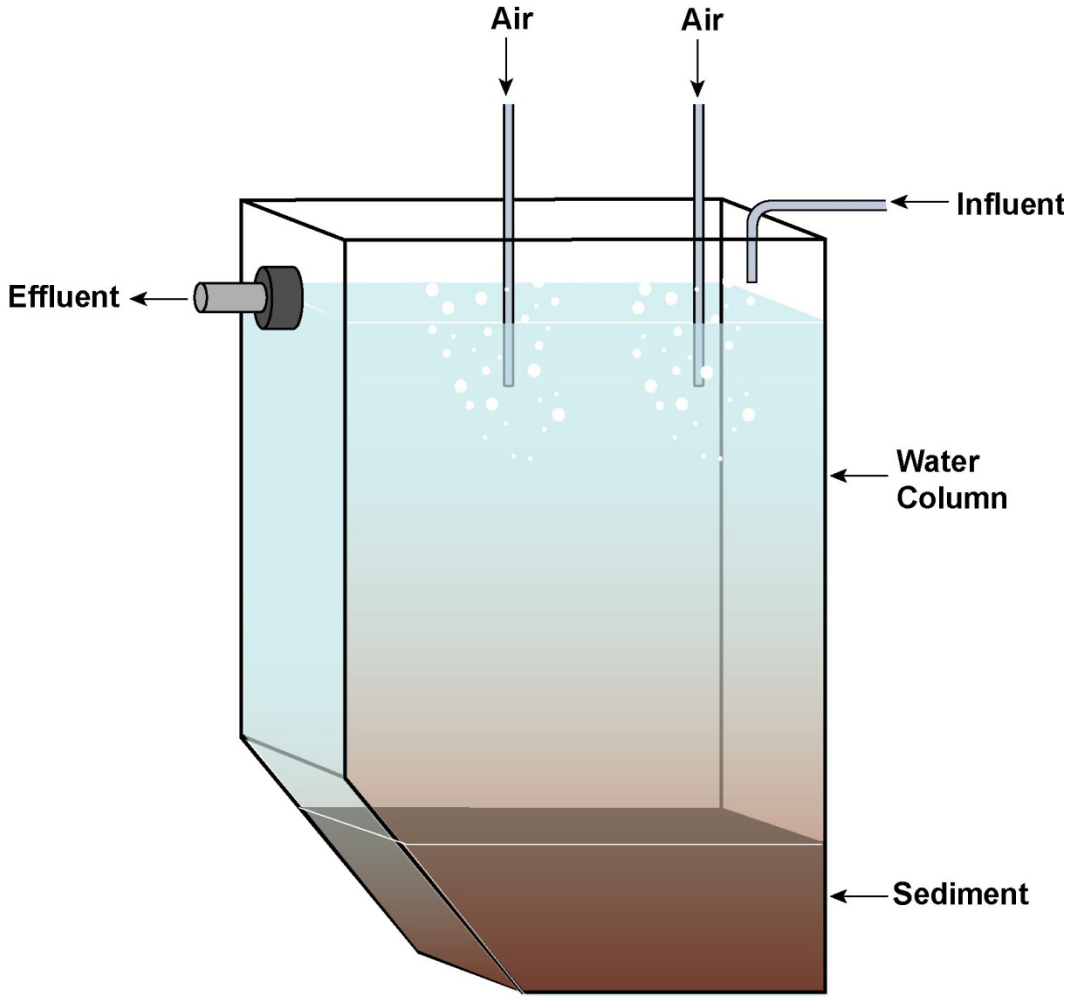


Figure 6.1. Schematic of continuous-flow system with DO concentration.

Table 6.1. Characteristics of the continuous-flow system feed.

Component	Feed Concentration	Effluent Concentration
pH	6.5 ± 0.2[a]	7.3 ± 0.2[a]
TSS, g/L	0.82 ± 0.02	0.03 ± 0.002
VSS, g/L	0.37 ± 0.01	0.03 ± 0.003
tCOD, mg/L	727 ± 1	127 ± 15
sCOD, mg/L	197 ± 27	58 ± 3
Chloride, mg Cl/L	116 ± 25	174 ± 30
Ammonia, mg N/L	30	ND[b]
Nitrate, mg N/L	ND[b]	6.4
Phosphate, mg P/L	10	5.7 ± 1.3
Sulfate, mg S/L	37.4 ± 2.3	45.5 ± 4.1

[a]Mean ± std. dev., n=3; [b] ND, not detected

evaluated by comparing the influent and effluent concentrations of sulfate normalized to chloride concentration.

The system performance was assessed by measuring TSS, VSS, tCOD, sCOD, chloride, ammonia, nitrate and sulfate in both the system feed and effluent. Evaporation from the system was significant; thus, in order to compensate for the water losses, the measured concentrations of all ions were normalized to the concentration of chloride for comparison between feed and effluent concentrations.

Phytosterol concentrations were measured in the system feed, effluent, sediment and water column. These concentrations were then used to assess phytosterol removal and distribution within the system. Feed and effluent samples were collected directly from the two streams. Water column samples were collected from the reactor after mixing the water column without disturbing the sediment. Sediment samples were collected with a long plastic tube mounted to a syringe. Gas produced by the sediment was collected by trapping gas bubbles in an inverted tube which was then sealed with a

rubber stopper. The gas was analyzed for composition and the data were used to help identify the microbial processes occurring in the sediment.

6.3. Results and Discussion

6.3.1. System Performance

DO was at or near saturation level throughout the water column and began to fall below saturation at the water/sediment interface. DO reached zero about 1 cm below the water/sediment interface. The DO profile indicates that anaerobic conditions existed within the reactor sediment and aerobic conditions existed in the water column. These characteristics make the continuous-flow reactor a suitable system for analyzing the partitioning and degradation of phytosterols within complex systems having multiple redox environments.

The steady-state sulfide concentration of the bulk sediment was 605 ± 171 mg S/L. Characteristics of the system effluent are given in Table 6.1. The chloride concentration increased by 49 % from influent to effluent as a result of water loss by evaporation from the reactor (Table 6.1). Sulfate only increased by 24 % which indicates that there was net removal of sulfate by the system. The lack of DO in the sediment, the production of metal sulfides and the net removal of sulfate show that sulfate-reducing conditions existed within the reactor sediment.

The gas produced in the sediment was composed of 40% methane, 42% nitrogen and 18% carbon dioxide; sulfide gas was not detected. It is postulated that precipitation of metal sulfides may have contributed to non-detectable sulfide gas. The gas composition shows that sulfate-reduction, methanogenesis and nitrate-reduction were significant processes occurring in the anaerobic sediment. Nitrate, which was not present

in the feed, was produced as a result of ammonia oxidation (i.e., nitrification) in the water column where the necessary aerobic conditions existed. Ammonia was completely absent from the system effluent. Nitrate was measured in the system effluent and accounted for 32 % of the ammonia nitrogen present in the feed after normalization to chloride concentration. The system removed 96 % of TSS, 92 % of VSS, 83 % of tCOD, and 70% of sCOD from the feed wastewater. Thus, this system achieved significant removal of suspended solids and COD from the untreated pulp mill wastewater. The system sediment and water column had a pH of 5.7 ± 0.1 and 7.8 ± 0.2, respectively. The pH of the system sediment was significantly lower than in the water column as a result of acid fermentation within the anaerobic sediment. VFAs were not detected in the sediment, but buildup of higher molecular weight organic acids probably contributed to the decreased pH. The sediment volume at steady-state was 1 L; thus, solids removal due to degradation in the sediment was occurring. The sCOD removal achieved by the continuous-flow system is greater than that achieved by the aerobic stock culture, indicating that anaerobic processes within the system contribute to removal of sCOD that is recalcitrant under purely aerobic conditions.

6.3.2. Phytosterol Distribution and Removal

Phytosterol concentrations in the feed, effluent, sediment and water column of the continuous-flow system were measured to assess the distribution and removal of phytosterols within the system (Table 6.2). About 87, 72 and 96% removal of campesterol, stigmasterol and β-sitosterol, respectively, was achieved across the system. The effluent stream had a very low solids concentration as a result of excellent (> 96%) solids removal. The water column contained biomass and wastewater solids which are

Table 6.2. Phytosterol concentrations (µg/L) in continuous-flow system compartments.

Stream/Compartment	Campesterol	Stigmasterol	β-sitosterol
Feed	14.6 ± 0.8[a]	17.6 ± 0.8	101.8 ± 2.5
Effluent	1.9 ± 0.7	4.9 ± 3.0	4.5 ± 1.2
Water Column	6.1	9.5	37.9
Sediment	1372.2 ± 54.2	1681.8 ± 370.4	7633.9 ± 1147.6

[a]Mean ± std. dev., n=2

not present in the effluent. The difference in phytosterol concentrations between the water column and effluent shows that the presence of solids, colloids, and dissolved organic matter resulted in phytosterol concentrations in the aerobic zone above those associated with only the aqueous phase (i.e., above expected aqueous solubility values). The three phytosterols accumulated to between 75 and 95 times their feed concentrations within the sediment. Thus, phytosterols are transported to the sediment as a result of the settling of solids, which were introduced with the wastewater or generated by the biological system. Once transported to an anaerobic environment, the phytosterols could not be biodegraded under the anaerobic conditions and accumulated. The three phytosterols were not degraded during anaerobic batch assays conducted under sulfate-reducing or methanogenic conditions as discussed in Chapter 5, sections 5.3.6 and 5.3.7, respectively.

A mass balance was performed for phytosterols in the continuous-flow system and used to calculate the expected concentration in the sediment, assuming no biotransformation of phytosterols occurred within the reactor. It was assumed that the concentration of phytosterols in the liquid phase throughout the water column and sediment is equal to the phytosterol concentration in the system effluent. The phytosterol

concentrations in the system influent, effluent and water column are assumed constant at the values given in Table 6.3 over the 14 month period. The evaporation rate from the system is assumed constant at 104 mL/d (33% of influent flow rate). The influent and effluent flow rates are therefore assumed to be 315 and 211 mL/d, respectively. The calculated mass of campesterol, stigmasterol and β-sitosterol which entered the system over the 14 months (426 days) of operation were 1959, 2361 and 13660 μg, respectively. The calculated mass of campesterol, stigmasterol and β-sitosterol which left the system over the same period were 171, 440 and 405 μg, respectively; thus, the mass of campesterol, stigmasterol and β-sitosterol remaining in the system (i.e. removed from the wastewater), assuming no biotransformation, were estimated to be 1788, 1921 and 13255 μg, respectively. The mass of campesterol, stigmasterol and β-sitosterol remaining in the system sediment and water column after 14 months were 1409, 1738 and 7861 μg, respectively. Thus, the mass of campesterol, stigmasterol and β-sitosterol biotransformed within the system over the 426 days of operation were 379, 183 and 5394 μg, which constituted 21, 9.5 and 41 %, respectively, of the total mass of each phytosterol removed from the wastewater during that time. The balance of the removed phytosterols remained in the system, where 97% of the total phytosterols were in the sediment. Based on data reported in Chapter 4 relative to the effect of pH on the desorption of phytosterols, because of the low pH in the sediment, the phytosterols are less likely to desorb from the solids once they settle than if they remained suspended in the water column.

Accumulation of phytosterols in aerobic sludge from pulp mill activated sludge units, at similar ranges to those observed in the continuous-floe system in the present study, has been reported previously (Mahmood-Kahn and Hall, 2003). The fact that

accumulation of phytosterols also occurred to such significant concentrations in aerobic sludge indicates that their bioavailability may be reduced once they become adsorbed to biosolids; however, this is in contrast to the significant biodegradation of phytosterols under aerobic conditions observed in batch assays performed in the present study (see Chapter 5). Increase of stigmasterol concentrations, reportedly observed across pulp mill ASBs (Cook et al., 1997), was not observed in the laboratory-scale system used in the present study.

6.4. Summary

Phytosterol distribution and removal within a continuous-flow biological treatment system simulating ASB systems was investigated. The continuous-flow system was derived from pulp mill ASB mixed liquor inoculum and fed untreated pulp mill wastewater to create a biological treatment environment similar to a pulp mill ASB. The system was run continuously for 14 months. Afterward, the distribution of phytosterols was assessed in the reactor sediment and water column and the removal of phytosterols from the wastewater was assessed by comparing the system feed and effluent constituents. The general wastewater treatment performance of the system was assessed by evaluating COD and SS removal.

The system developed a steady-state sediment volume of 1 L from accumulated wastewater solids and biomass. Sulfate-reduction, nitrate-reduction, and methanogenesis took place in the sediment. The DO profile of the reactor showed that the water column was at near saturation DO levels until the water/sediment interface. DO rapidly dropped below the interface, and the sediment became completely anaerobic below about 1 cm depth. The system achieved significant wastewater tCOD and TSS removal, 83% and

96%, respectively. The system also achieved 72 to 96% removal of individual phytosterols from the pulp mill wastewater with removal of 23, 14 and 41 % of campesterol, stigmasterol and β-sitosterol, respectively, being attributed to biodegradation. Phytosterols remaining in the system were mainly associated with the sediment (97 %), where low pH and anaerobic conditions contribute to a low potential for phytosterol desorption and/or biodegradation.

CHAPTER 7

CONCLUSIONS AND RECOMMENDATIONS

Phytosterols are known endocrine disrupting compounds naturally produced by plants, found in natural and engineered aquatic systems. Despite being ubiquitous and problem-causing, very little is known about their chemical and physical properties or their biotransformation potential. The research presented here sought to evaluate several important physical properties of phytosterols and assess their biotransformation potential under aerobic, nitrate-reducing, sulfate-reducing and methanogenic conditions. A pulp mill ASB was chosen as a model biological treatment system. Batch cultures and a continuous-flow system were developed from ASB mixed liquor and sediment. Based on the conducted research, several conclusions could be reached:

1) Aqueous solubility at 22°C was below 10 μg/L for cholesterol and below 1 μg/L for individual phytosterols present as a mixture.
2) Cholesterol had about a two-fold higher affinity for adsorption to aerobic biomass than stigmasterol. The affinity of both sterols for biomass solids decreased as the total sterol concentration increased.
3) Cholesterol exhibited about a four-fold greater affinity for adsorption to wastewater solids than stigmasterol at pH 7. The affinity of stigmasterol for adsorption to wastewater solids decreased two-fold as pH increased from 7 to 10. The affinity of the sterols for pulp mill wastewater solids was 2 orders of magnitude less than for aerobic biomass and did not change with increasing sterol concentration.

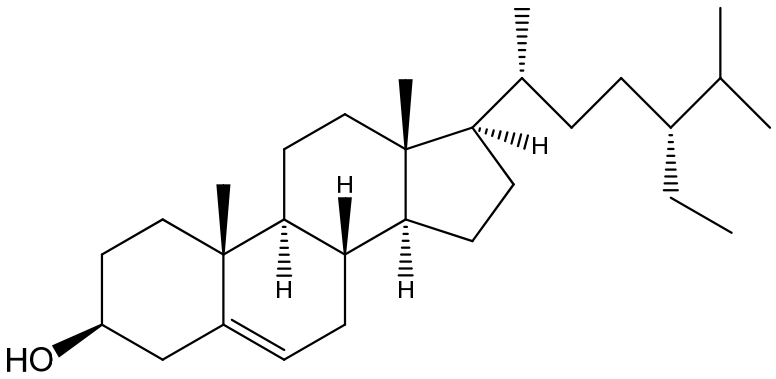
4) Desorption rate and extent for stigmasterol from pulp mill wastewater solids was pH-dependent. Desorption showed two-phase behavior: an initial rapidly desorbing fraction and a second, slowly desorbing fraction. Initial desorption rate was similar at pH 5 and 7; however, increasing pH to 9 increased the desorption rate and extent during the initial phase. The extent of desorption after 9 successive desorption steps varied from 16 to 55% at pH 5 and 9, respectively.
5) Phytosterols and cholesterol were biotransformed by an aerobic mixed culture under aerobic conditions at pH 7 and 22°C. The biotransformation rate was the same whether ammonia or nitrate was used as the inorganic nitrogen source. Biotransformation of phytosterols under aerobic conditions was induced by the presence of an easily degradable carbon source indicating that degradation is the result of either co-metabolism or secondary metabolism.
6) The results of an assay performed with an aerobic culture under nitrate-reducing conditions at pH 7 and 22°C showed a decrease in phytosterols concentrations, but biotransformation could not be conclusively shown.
7) Mixed sulfate-reducing and methanogenic cultures at pH 7 and 22°C were not able to biotransform phytosterols under sulfate-reducing and methanogenic conditions, respectively.
8) A continuous-flow system was effective at removing 72 to 96% of individual phytosterols from the pulp mill wastewater stream. Biotransformation accounted for 21, 9.5 and 41 % of campesterol, stigmasterol and β-sitosterol removal, respectively. Phytosterols accumulated in the system sediment and accounted for 97 % of the total phytosterols remaining in the system.

Overall, it was determined that phytosterols are biodegradable under aerobic conditions but are recalcitrant under sulfate-reducing and methanogenic conditions. The phase distribution of phytosterols is dependent on system pH, with desorption extent increasing with increasing pH. Significant phytosterol removal from pulp mill wastewater is possible from a combination of biotransformation and partitioning to wastewater solids and biosolids. Once phytosterols are adsorbed onto solids, they may be transported, under quiescent conditions, to bottom sediments where low pH and anaerobic conditions contribute to phytosterol sequestration. Control of pH, DO and available carbon and energy sources in biological treatment systems can enhance phytosterol removal.

Additional phase distribution work is recommended to determine the intrinsic aqueous solubility of individual phytosterols and the phase partitioning behavior of phytosterols in the presence of dissolved organic matter. These processes affect the bioavailability of phytosterols by facilitating their dissolution into aquatic systems. Further investigation into the metabolic processes involved during aerobic biotransformation of phytosterols is recommended to determine if the process is co-metabolic or the result of secondary metabolism requiring a degradable, primary substrate. Substrate requirements for phytosterol degradation would be applicable to designing effective treatment systems. The biotransformation potential of phytosterols under nitrate-reducing conditions by a pulp mill treatment system derived culture must be investigated further to determine if this is a feasible treatment process.

APPENDIX A

GIBB'S FREE ENERGY OF STEROLS



Cholesterol

Group	# of Occurrence	Contribution (kcal/mol)	Total Contribution (kcal/mol)
Origin	1	-23.6	-23.6
$-CH_3$	5	7.9	39.5
-OH	1	-32.0	-32.0
<Open Chain>			
-CH<	3	-4.8	-14.4
$-CH_2-$	2	1.7	3.4
<One Ring>			
$-CH_2-$	8	6.1	48.8
-CH<	2	-2.2	-4.4
=CH-	1	9.6	9.6
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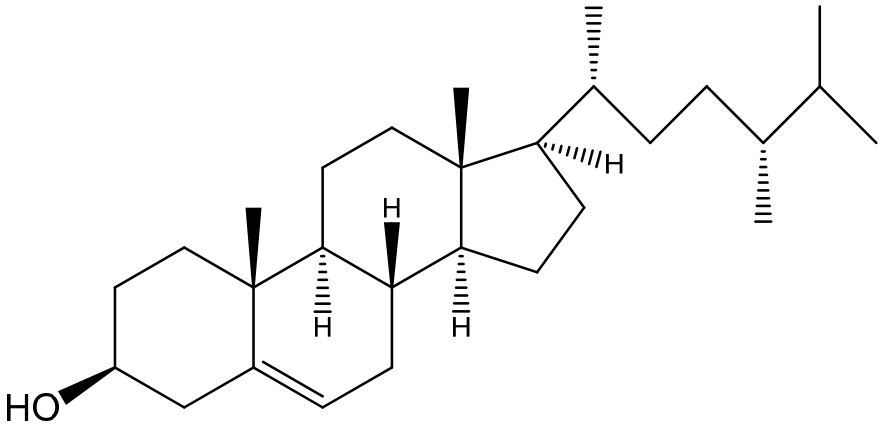
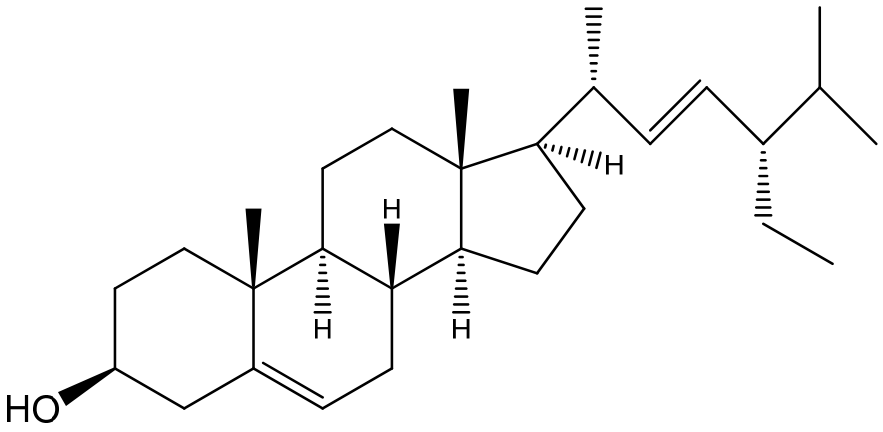
>C<	2	-12.0	-36.0
=C<	1	16.8	16.8
-CH<	3	-2.2	-6.6
ΔG^{o}'			**13.1 (kcal/mol)** **54.8 (kJ/mol)**



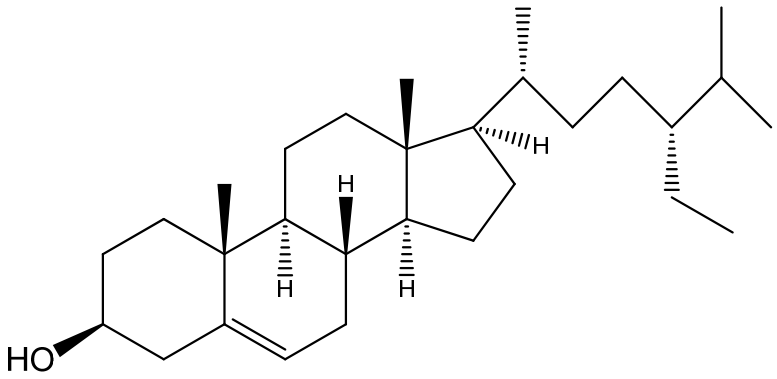
Campesterol

Group	# of Occurrence	Contribution (kcal/mol)	Total Contribution (kcal/mol)
Origin	1	-23.6	-23.6
$-CH_3$	6	7.9	47.4
-OH	1	-32.0	-32.0
<Open Chain>			
-CH<	3	-4.8	-14.4
$-CH_2-$	2	1.7	3.4
<One Ring>			
$-CH_2-$	8	6.1	48.8
-CH<	2	-2.2	-4.4
=CH-	1	9.6	9.6
<Two Rings>			
>C<	2	-12.0	-36.0
=C<	1	16.8	16.8
-CH<	3	-2.2	-6.6
$\Delta G^{o'}$			**21.0 (kcal/mol)** **87.9 (kJ/mol)**



Stigmasterol

Group	# of Occurrence	Contribution (kcal/mol)	Total Contribution (kcal/mol)
Origin	1	-23.6	-23.6
$-CH_3$	6	7.9	47.4
-OH	1	-32.0	-32.0
<Open Chain>			
=CH-	2	11.1	22.2
-CH<	3	-4.8	-14.4
$-CH_2-$	1	1.7	1.7
<One Ring>			
$-CH_2-$	8	6.1	48.8
-CH<	2	-2.2	-4.4
=CH-	1	9.6	9.6
<Two Rings>			
>C<	2	-12.0	-36.0
=C<	1	16.8	16.8
-CH<	3	-2.2	-6.6
ΔG^{o}'			**41.5 (kcal/mol)** **173.6 (kJ/mol)**



β-sitosterol

Group	# of Occurrence	Contribution (kcal/mol)	Total Contribution (kcal/mol)
Origin	1	-23.6	-23.6
$-CH_3$	6	7.9	47.4
-OH	1	-32.0	-32.0
<Open Chain>			
-CH<	3	-4.8	-14.4
$-CH_2-$	3	1.7	5.1
<One Ring>			
$-CH_2-$	8	6.1	48.8
-CH<	2	-2.2	-4.4
=CH-	1	9.6	9.6
<Two Rings>			
>C<	2	-12.0	-36.0
=C<	1	16.8	16.8
-CH<	3	-2.2	-6.6
$\Delta G^{o'}$			**22.7 (kcal/mol)** **95.0 (kJ/mol)**

APPENDIX B

STANDARD GIBB'S FREE ENERGY OXIDATION REACTIONS

AT PH 7 AND 22°C

Campesterol

Redox Condition	$\Delta G_r'$ kJ/eeq
Aerobic $C_{28}H_{48}O + 39.5O_2 \leftrightarrow 28CO_2 + 24H_2O$	-104.99
Nitrate-reducing $C_{28}H_{48}O + 31.6NO_3^- + 31.6H^+ \leftrightarrow 28CO_2 + 39.8H_2O + 15.8N_2$	-98.47
Sulfate-reducing $C_{28}H_{48}O + 19.75SO_4^{2-} + 29.625H^+ \leftrightarrow 28CO_2 + 24H_2O + 9.875H_2S + 9.875HS^-$	-5.42
Methanogenic $C_{28}H_{48}O + 15.5H_2O \leftrightarrow 8.25CO_2 + 19.75CH_4$	-2.74

Stigmasterol

Redox Condition	**ΔG_r' kJ/eeq**
Aerobic $C_{29}H_{48}O + 40.5O_2 \leftrightarrow 29CO_2 + 24H_2O$	-105.31
Nitrate-reducing $C_{29}H_{48}O + 32.4NO_3^- + 32.4H^+ \leftrightarrow 29CO_2 + 40.2H_2O + 16.2N_2$	-98.79
Sulfate-reducing $C_{29}H_{48}O + 20.25SO_4^{2-} + 30.375H^+ \leftrightarrow 29CO_2 + 24H_2O + 10.125H_2S + 10.125HS^-$	-5.74
Methanogenic $C_{29}H_{48}O + 16.5H_2O \leftrightarrow 8.75CO_2 + 20.25CH_4$	-3.06

Cholesterol

Redox Condition	ΔG_r' kJ/eeq
Aerobic $C_{27}H_{45}O + 37.75O_2 \leftrightarrow 27CO_2 + 22.5H_2O$	-104.71
Nitrate-reducing $C_{27}H_{45}O + 30.2NO_3^- + 30.2H^+ \leftrightarrow 27CO_2 + 37.6H_2O + 16.4N_2$	-98.19
Sulfate-reducing $C_{27}H_{45}O + 18.875SO_4^{2-} + 28.3125H^+$ $\leftrightarrow 27CO_2 + 22.5H_2O + 9.4375H_2S + 9.4375HS^-$	-5.14
Methanogenic $C_{27}H_{45}O + 15.25H_2O \leftrightarrow 8.125CO_2 + 18.875CH_4$	-2.46

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